

IMPORTANT ANNOUNCEMENT

Due to the increased flow of excellent papers from research workers throughout the world, *Journal of Chemical Ecology* will be expanding in 1989, with the number of pages published *increased by about 25%*. Thus, the subscription price for Volume 15, 1989 (12 issues) is \$345.00 (outside the U.S., \$405.00). The price for individuals certifying that the journal is for their personal use is \$72.50 (outside the U.S., \$89.00).

OLFACTORY ORIENTATION RESPONSES BY WALKING FEMALE *Ips paraconfusus* BARK BEETLES I. Chemotaxis Assay

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Abstract—Gas-liquid chromatography of the air within the arena developed for this assay showed that a concentration gradient was established within 1–2 min of applying the pheromone (ipsenol, ipsdienol, *cis*-verbenol), and that this gradient was nearly constant for 20–95 min after application. The concentration fell rapidly and approximately exponentially between the source and the center of the arena. Turning rate and the number of beetles that reached the source increased, and heading with respect to the source decreased, in the presence of pheromone. Responses of beetles that did and did not reach the source were significantly different, but within each group there were no significant differences among dosages. Turning rate and heading varied little with distance from the source, while walking rate decreased as distance from the release point of the beetles increased. We hypothesize that dosage exerts its major effect on source location by altering the probability that a beetle will enter into orientation behavior and that beetles orienting to sources have similar behaviors even when orienting to a wide range of dosages.

Key Words—*Ips paraconfusus*, Coleoptera, Scolytidae, bark beetles, taxis, chemotaxis, orientation, olfaction, pheromones.

INTRODUCTION

These experiments were undertaken while developing assays for a study of quality discrimination among the components of a multicomponent pheromone

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(Akers and Wood, 1989). An assay had to provide clear, differential responses to olfactory stimuli, which could serve as a behavioral basis for defining different odor qualities. The extent to which an assay met this requirement was tested by exposing beetles to a dosage series of a standard pheromone blend. *Ips paraconfusus* females readily found pheromone sources 16–18 cm away in still air, and we became curious as to the mechanisms involved. A complete knowledge of the behavioral mechanism was not necessary to use the assay as an indicator of olfactory significance, but the more complete the behavioral description, the better we can visualize possible neural mechanisms. This, in turn, should assist in interpreting neurophysiological recordings and in deriving models from them (Kein 1974ab, 1975).

Several other studies of orientation in still air suggested that the paths of insects become more linear as they near a source of odor (Klingler, 1958; Bell and Tobin, 1981; Fraenkel and Gunn, 1961, Chap. 18). Since higher dosages should produce steeper concentration gradients (Crank, 1956), the inference might be drawn from these previous studies that as dosage increased all the insects in a population would shift their overall directional vectors (Bell and Tobin, 1982) towards the source and then approach it in a more linear or direct manner. The proportion of beetles that reached the source would increase as the intersections of their paths with the arena wall became more narrowly distributed around the source. Alternatively, the group that reaches the source might be comprised of beetles that enter into orientation behavior while the group that does not reach the source might be comprised of beetles that do not enter into orientation. Dosage would then change the proportion of orienting beetles, while the behavior of orienting beetles in different dosages might be similar. In our first analyses the beetles appeared to head more directly to the source as dosage increased, which seemed to support the first hypothesis. However, when the second hypothesis occurred to us, a more extensive analysis indicated that it was probably the more correct of the two. The initiation of orientation therefore appears to have a strong all-or-none component. A second paper (Akers, 1989) examines the mechanisms by which an orienting beetle may reach the source.

METHODS AND MATERIALS

Compounds. The attractant pheromone of *I. paraconfusus* is a blend of ipsenol (Ip), ipsdienol (Id), and *cis*-verbenol (cV) (Silverstein et al., 1966; Wood et al., 1967, 1968). The compounds were obtained from Booregard Industries, Ltd., Sarpsborg, Norway. Gas-liquid chromatography (GLC) showed the following purities: ipsenol 95%, ipsdienol 98%, and *cis*-verbenol >99%. None of the compounds had any contamination of the other compounds. A small

sample of the ipsenol was purified by GLC to >99% purity. This sample was tested in the chemotaxis assay and was found to have full activity.

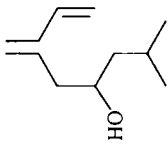
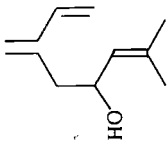
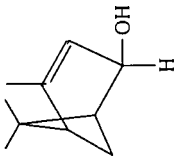
The Chemotaxis Assay. The design of the assay followed discussions in Fraenkel and Gunn (1961, pp. 271–284) and was intended to create a concentration gradient across an arena. The top and bottom of the arena were each a pane of double-strength glass, 40.6 cm square and sealed along the edges. The top had a 1-cm hole in the center, cut with a diamond-dust core drill (Equamat Distribution, Santa Clara, California). The wall of an arena was a ring of polyvinyl chloride plastic, 1 cm high, 40 cm OD, 39 cm ID, with silicon rubber gaskets permanently bonded to the edges of the wall. To assemble an arena, a wall was centered between an upper and lower pane of glass, and the edges were bound with heavy rubber bands. The hole in the upper pane was sealed with a stopper except when access to the interior was needed. A treatment was placed on a 22-mm-square cover slip. Once the solvent evaporated within a fume hood, the cover slip was slipped under the wall of an assembled arena, and placed in a standardized location just inside the wall.

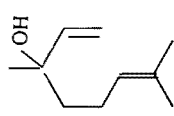
The spatial and temporal variation of the pheromone within the arena were estimated by taking samples of air directly from an arena with a gas-tight syringe and injecting them onto a gas-liquid chromatograph (GLC) fitted with flame ionization detectors. Peak areas were converted to quantity by comparing them to peaks produced by known amounts of linalool. The amount of compound remaining behind in the syringe after injection was not estimated. Therefore, only the amount recovered and not the actual concentration in the air is reported. Linalool, an isomer of ipsenol, was used in these studies to conserve pheromonal compounds. The molecular weight, structure, and adjusted boiling points of ipsenol, ipsdienol, and linalool are very similar (Table 1). In addition, linalool and ipsenol are indistinguishable on many GLC columns (Young et al., 1973). One milligram of linalool applied to an arena gave a concentration in the air similar to 1 mg of ipsdienol, ipsenol, or *cis*-verbenol. Accordingly, linalool appeared to be a reasonable substitute for the pheromonal compounds.

The first experiment evaluated the behavior of the concentration gradient within the arena through time. One milligram of linalool was applied to the dispenser, and 1-ml samples of air were taken periodically (Figure 1) over the dispenser and at the center of the arena. A difference in concentration appeared almost immediately between the center and the dispenser. The concentration difference was nearly constant between 20 min and 95 min after charging the arena. Diffusion periods of 30 or 60 min were thereafter used in all experiments, and all beetles in a single arena were run by 15 min after the diffusion period.

The spatial variation within the arena was further characterized at the 60-min diffusion time by taking 1-ml samples of air at four points along the line between the dispenser and the center of the arena (Figure 2), by fitting the

TABLE I. PHYSICAL PROPERTIES OF PHEROMONAL COMPOUNDS AND THEIR BEHAVIOR IN ASSAYS

Compound	Chemical name	Formula	Structure	MW	Boiling point	Concentration in air ^a (ng/ml air)		Kinesis assay
						Source	Center	
Ipsenol	2-methyl-6-methylene-7-octen-4-ol ^b	C ₁₀ H ₁₇ O		153	190-193 ^c	52	6.2	4.3
Ipsdienol	2-methyl-6-methylene-2,7-octadien-4-ol ^b	C ₁₀ H ₁₆ O		152	202 ^d	31	3.6	9.5
cis-Verbenol	2-pinen-4-ol ^c	C ₁₀ H ₁₆ O		152	NA	36	4.6	7.8

Linalool	3,7-dimethyl-1,6-octadien-3-ol ^a	$C_{10}H_{18}O$		154	198-200 ^c	46	5.8	4.7
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^aDosage was 1 mg/arena. Response is mean of three arenas.

^bSilverstein et al. (1966).

^cMori et al. (1979). Corrected to standard temperature and pressure using corrections of Hass and Newton (1980).

^dMori (1974). Corrected to standard temperature and pressure using corrections of Hass and Newton (1980).

^eCRC Handbook of Chemistry and Physics (1980).

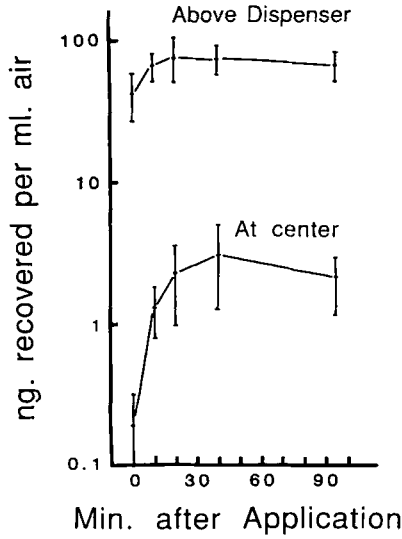


FIG. 1. The concentration of linalool in the air above the pheromone dispenser and at the center of the arena, near the release point of the beetles. One milligram was applied approx. 1 min before the first sample was taken at time 0. Distance between the center and the dispenser was approx. 17 cm. Error bars show standard deviations.

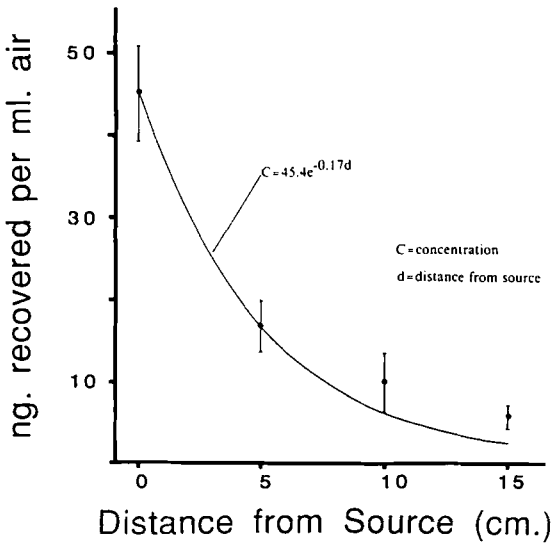


FIG. 2. The concentration of linalool in the air at various distances between the dispenser and the center of the arena. One milligram was applied to the dispenser 60 min before taking the samples. Error bars show standard deviations.

syringe with a long needle. The dispenser contained 1 mg of linalool. All samples from a single arena were taken within a 5-min period. Five arenas were sampled. The theory of diffusion indicates that, if a material diffuses freely in two dimensions without any constraints, its concentration should fall off exponentially with the square of the distance from the source (Crank, 1956). In this experiment, the relationship was estimated as falling off exponentially with the distance, not its square. When both forms of relationships were fitted to the data using the nonlinear regression (NLIN) procedure available in the Statistical Analysis System (Goodnight and Sall, 1982), the relationship based on the distance produced the lower sums of squares.

Observation and Recording Procedures. Preliminary experiments in the open laboratory indicated that the beetles were not orienting at random within untreated arenas (Akers, 1985). Accordingly, an observation chamber was developed that isolated the insects from these possibly confounding cues (Figure 3). The beetles oriented at random within the chamber when no odor cues were available. All experiments were performed using the chamber in the darkened laboratory.

The movements of a beetle were recorded by marking its position by hand at 1-sec intervals, with reference to an electronic metronome. The beetle's path was usually traced at the same time with the free hand. If an animal stopped walking, recording of its position ceased until it began moving again. However, the beetles are highly thigmotactic, and most did not stop moving until they contacted the wall of the arena or the pheromone source, at which time tracking ceased. Tracks were later entered into a computer via a digitizing table. The observation procedure was able to separate trails differing in average linear speeds by 0.02–0.04 cm/sec and in turning rates by about 2–5°/sec (Akers, 1985).

Handling of Experimental Animals. Naturally infested ponderosa pine was collected as logging debris, mostly from the vicinity of Blodgett Research Forest near Georgetown, Eldorado County, California. Infested logs were held at 5°C until needed. At such time, the logs were moved to a rearing chamber in a greenhouse, where the emerging beetles were attracted into a refrigerator at 2–5° C (Browne, 1972). Beetles were stored for periods up to three weeks before use, with no signs of adverse effects (Borden, 1967).

One day before they were to be used, the beetles were tested for their ability to perform in the assay. They were placed on a flat piece of glass and were accepted for assays if they had both their antennae and could walk more than 5–6 cm upon the glass. The sex of the selected beetles was then determined. If the beetles were kept uncrowded after emergence from the log, they did not damage one another, and a high proportion of the females were usable. Selected females were stored in individual glass tubes. They were removed from the cold 5–20 min before use. Recovery from the cold took a minute or

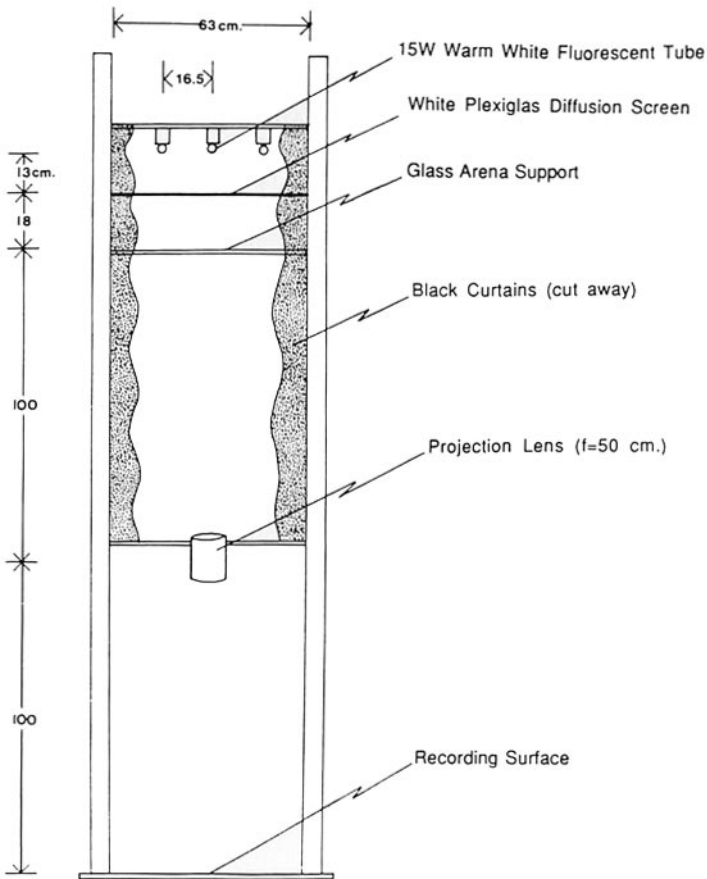


FIG. 3. The observation chamber used in all experiments to isolate the beetles from extraneous cues for orientation.

two at room temperature, which was $22 \pm 1^\circ\text{C}$. They were kept cool until a few minutes before use, since their struggles within the storage tubes seemed to decrease their vigor after an hour or so. A beetle was brought out of the cold directly onto the observation platform of the observation chamber, where it could recover in the same light and temperature conditions under which it would be tested. A beetle was used only once.

When a beetle was to be released, the tape was removed from the storage tube and the beetle was slid gently but immediately into an L-shaped, glass release tube inserted through the hole in the arena. The outlet of the tube was oriented at approx. 90° with respect to the source.

Dosage Series. The experimental design was a dosage series of a 1:1:1 blend of the three pheromonal compounds, as used in earlier behavioral tests (Byers and Wood, 1981). Dosages differed by powers of 10, had a range of 10^{-4} –1 mg of each compound, and were dissolved in 0.1 ml of pentane. The series was run with diffusion periods of both 30 and 60 min. Solutions were stored at -60°C or over Dry Ice until a few minutes before use.

In order to minimize contamination between treatments, each treatment had its own arena. An arena was rinsed after each use in hot (approx. 70°C) water, since the pheromone compounds are moderately soluble in water. The water flowed at a rate that provided an exchange of water approximately every 4 min. The rinse lasted about an hour. The parts of an arena were then aired for two to five days before the next use.

A preliminary experiment indicated that the beetles did not exhibit any trail-following behavior or otherwise interact significantly with one another when more than one beetle was released within an arena (Akers, 1985). In this experiment, the relationships between the tracks of two beetles that had run together within a single arena were compared to the relationships between the tracks of a pair of beetles that had run in separate arenas. The average distance that the second beetle of a pair maintained with respect to the trail of the first beetle was measured, as were the walking and turning rates of the second beetle. There were no significant differences in these measurements between the beetles that had the opportunity to follow a trail and those that did not. Observations made on different beetles moving within a single arena could therefore be considered as independent for statistical purposes, and running more than one beetle within a single arena greatly reduced the time required per replicate. Five beetles were usually run successively in each arena, with an interval of approx. 2–3 min between runs. A randomized block design was used as a precaution, in case the supply of beetles was lost during an experiment, but the effects of blocks were slight and are not reported. The experimenter did not know the identity of the treatments, either during the experiment or during the manipulation of the data.

Analysis of Orientation Data. The kinetic responses measured were orthokinesis, or linear rate of motion, and klinokinesis, or rate of turning. The estimate of the rate of motion was the mean distance between each point on a beetle's track. Two related measures were used to summarize the turning rate. Both depended on measuring the turn angle at each point on a track. The turn angle at a point was defined as the angle between the direction from the previous to the current point on the track and the direction from the current to the next point. Zero degrees was defined as straight ahead from the current point on the track. For the net turning rate, the "handedness" of a turn was taken into consideration, with left-hand turns defined as positive. In the gross turning rate, the absolute value of each turn was used. The estimates of the rates were the mean turn angles over all the points on the track. A heading was also determined

at each point on an insect's track by finding the angle between the direction towards the source and the direction from the present point to the next point on the track. Zero degrees was defined as straight towards the source. Both a mean net and a mean gross heading were defined.

The statistical analyses available for circular variables are more complex and much more limited than those available for linear variables (Batschalet, 1965, 1981). However, the gross angles used here are defined on only half the circle and thus are actually angular distances. As such they may be treated as linear variables (Batschalet, 1981, p. 231). On the other hand, the net angles are defined on the full circle and thus are true circular variables. The mean angle and its angular (=standard) deviation may be estimated from the mean vector (Batschalet, 1981, Chap. 1). This provides an unbiased estimate of the mean, but a fairly biased estimate of the angular deviation (Batschalet, 1981, p. 46). However, once the mean net angle of a track is obtained, the major point of interest in comparing means of beetles and treatments is the magnitude of the mean, which is again an angular distance. The summaries made then were: walking rate and its variation within a track, net turning rate, gross turning rate and its variation within a track, mean net heading, and mean gross heading and its variation within a track.

RESULTS

A logistic regression showed that dosage had a significant effect on the number of beetles that reached the source (Figure 4) (30-min diffusion period: $a = 1.42$, $B = 0.58$; 60-min diffusion period: $a = 2.22$, $B = 0.86$. All parameters are significantly different from 0 at $P < 0.001$). Increasing dosage decreased the net and gross headings (Table 2, all beetles; Figures 5F, G; 6F, G), implying that beetles headed more directly towards sources of higher dosage. Increasing dosage also increased the gross turning rate (Figures 5C, 6C). It decreased the walking rate in the 60-min diffusion period (Table 2: dosage, all beetles; Figure 6A), but the effect was not significant in the 30-min diffusion period (Table 2: dosage). There was no significant effect on the magnitude of the net turning rates.

Since turning rate increased with dosage while walking rate either decreased or remained constant, turning radius therefore decreased with increasing dosage, meaning that the animals made tighter turns in the higher dosages. Such behavior was difficult to reconcile with the notion that the tracks of the beetles were more linear in the higher dosages. Further, a comparison of trails of beetles that did (Figure 7: A_1 , B_1) and did not (Figure 7: A_2 , B_2) reach the source

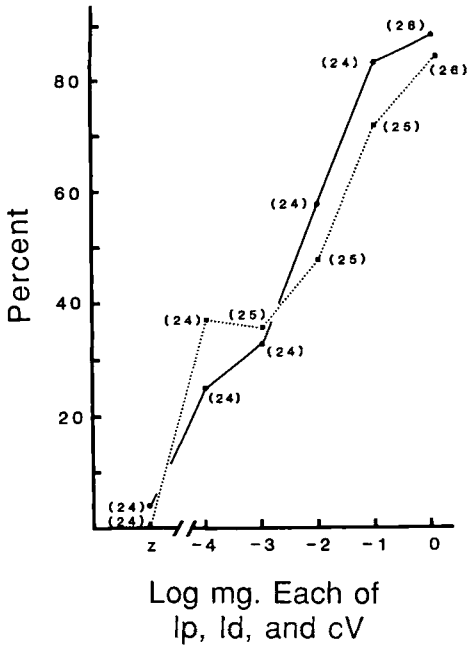


FIG. 4. The effect of pheromone dosage on the proportion of beetles that reached the pheromone source. Solid line represents experiment with 60-min diffusion period and dotted line represents experiment with 30-min diffusion period. Numbers in parentheses are sample sizes for respective treatments. cV = *cis-verbenol*, I_d = *ipsdienol*, I_p = *ipsenol*, $z = 0$ dosage level.

suggested that the behavior of the beetles differed fairly strongly between the two groups. Samples of trails of beetles that reached the source looked similar, even though the beetles were exposed to different dosages (Figure 7: A_1 , B_1). Therefore, the behavior of beetles that did and did not reach the source was compared.

The mean gross headings were significantly different between the two groups (Table 2: *rs* vs. *nrs*; Figures 5G, 6G). Beetles that did not reach the source had mean gross headings that did not differ among dosages (Table 2: dosage, *nrs*; Figures 5G, 6G). Beetles that reached the source also had mean gross headings that did not differ among dosages (Table 2: dosage, *rs*; Figures 5G, 6G), even though these beetles might have been expected to approach higher dosages more directly.

Similar results were apparent from other behavioral responses. Walking

TABLE 2. ANALYSES OF VARIANCE FOR EFFECTS OF DOSAGE AND WHETHER BEETLES LOCATED THE SOURCE ON BEHAVIORAL RESPONSES

Behavioral response	Comparison between	Diffusion period (min)	Number of beetles	F_{obs}	P value ^a
Walking rate	Dosages, all beetles	30	149	1.16	0.330
		60	146	3.54	0.005
	rs vs nrs ^b	30	125	26.20	<0.0001
		60	122	19.42	<0.0001
	Dosages, rs	30	70	0.16	0.959
		60	73	0.36	0.835
	Dosages, nrs	30	55	1.26	0.299
		60	49	1.78	0.150
Standard deviations of walking rates	Dosages, all beetles	30	149	0.55	0.737
		60	146	3.03	0.013
	rs vs. nrs	30	125	19.97	<0.0001
		60	122	4.82	0.030
	Dosages, rs	30	70	1.48	0.219
		60	73	1.70	0.160
	Dosages, nrs	30	55	1.39	0.250
		60	49	1.38	0.256
Gross turning rate	Dosages, all beetles	30	149	5.00	0.0003
		60	146	13.82	<0.0001
	rs vs. nrs	30	125	104.8	<0.0001
		60	122	71.8	<0.0001
	Dosages, rs	30	70	1.57	0.194
		60	73	5.76	0.0005
	Dosages, nrs	30	55	0.52	0.723
		60	49	0.60	0.663
Standard deviations of gross turning rates	Dosages, all beetles	30	149	2.72	0.022
		60	146	10.36	<0.0001
	rs vs. nrs	30	125	114.40	<0.0001
		60	122	59.73	<0.0001
	Dosages, rs	30	70	0.75	0.559
		60	73	3.95	0.006
	Dosages, nrs	30	55	0.55	0.700
		60	49	0.56	0.693
Magnitudes of net turning rates	Dosages, all beetles	30	149	1.08	0.373
		60	146	0.29	0.915
	rs vs. nrs	30	125	0.81	0.371
		60	122	0.71	0.402
	Dosages, rs	30	70	0.87	0.487
		60	73	0.16	0.957
	Dosages, nrs	30	55	1.50	0.217
		60	49	0.62	0.648

TABLE 2. Continued

Behavioral response	Comparison between	Diffusion period (min)	Number of beetles	F_{obs}	P value ^a	
Mean gross heading	Dosages, all beetles	30	149	3.74	0.003	
		60	146	6.61	<0.0001	
	rs vs. nrs	30	125	312.1	<0.0001	
		60	122	234.5	<0.0001	
	Dosages, rs	30	70	1.85	0.130	
		60	73	0.99	0.418	
Dosages, nrs	30	55	0.66	0.623		
	60	49	0.07	0.990		
Standard deviations of mean gross headings	Dosages, all beetles	30	149	1.02	0.412	
		60	146	0.61	0.698	
	rs vs. nrs	30	125	11.05	0.001	
		60	122	5.15	0.025	
	Dosages, rs	30	70	1.30	0.278	
		60	73	0.79	0.535	
	Dosages, nrs	30	55	1.14	0.348	
		60	49	0.44	0.776	
	Magnitudes of net headings	Dosages, all beetles	30	149	4.30	0.001
			60	146	3.86	0.003
rs vs. nrs		30	125	501.6	<0.0001	
		60	122	355.6	<0.0001	
Dosages, rs		30	70	2.82	0.032	
		60	73	1.39	0.247	
Dosages, nrs		30	55	0.19	0.942	
		60	49	1.05	0.390	

^aThe P value is the probability of obtaining an $F_{(\text{obs})}$ greater than the observed F , given that H_0 is true. H_0 : means being compared are not different.

^brs = beetles which reached the source. nrs = beetles which did not reach the source. Control beetles were dropped from analyses where the population was divided into those that did and did not reach the source.

rate was lower among beetles that reached the source than among beetles that did not (Table 2: rs vs. nrs; Figures 5A, 6A). There were no significant differences among dosages in either group (Table 2: dosage, nrs, and dosage, rs; Figures 5A, 6A). The results for gross turning rate were somewhat less clear. In either diffusion period, beetles that reached the source had higher turning rates than beetles that did not (Table 2: nrs vs. rs; Figures 5C, 6C). In the

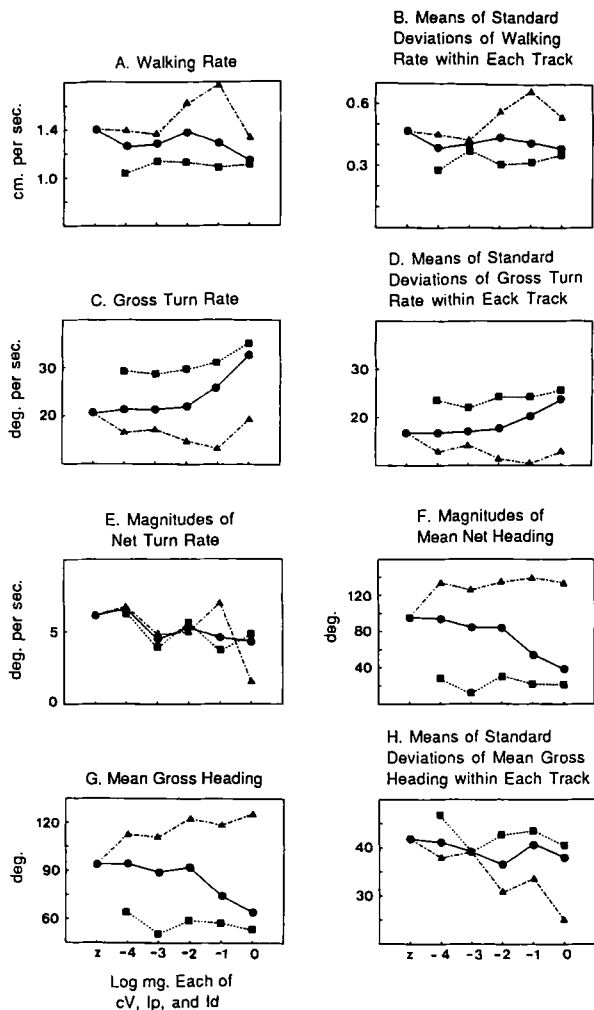


FIG. 5. Comparison of behavioral measurements for beetles that did and did not reach the source of pheromone in the experiment with the 30-min diffusion period. Solid circles indicate response of whole population; squares indicate response of beetles that reached source; and triangles indicate response of beetles that did not reach source.

experiment with the 30-min diffusion period, there were no significant differences among dosages in either group. In the experiment with a 60-min diffusion period, there were no significant differences among dosages for the beetles that did not reach the source (Table 2: dosage, nrs; Figure 6C), but turning rate

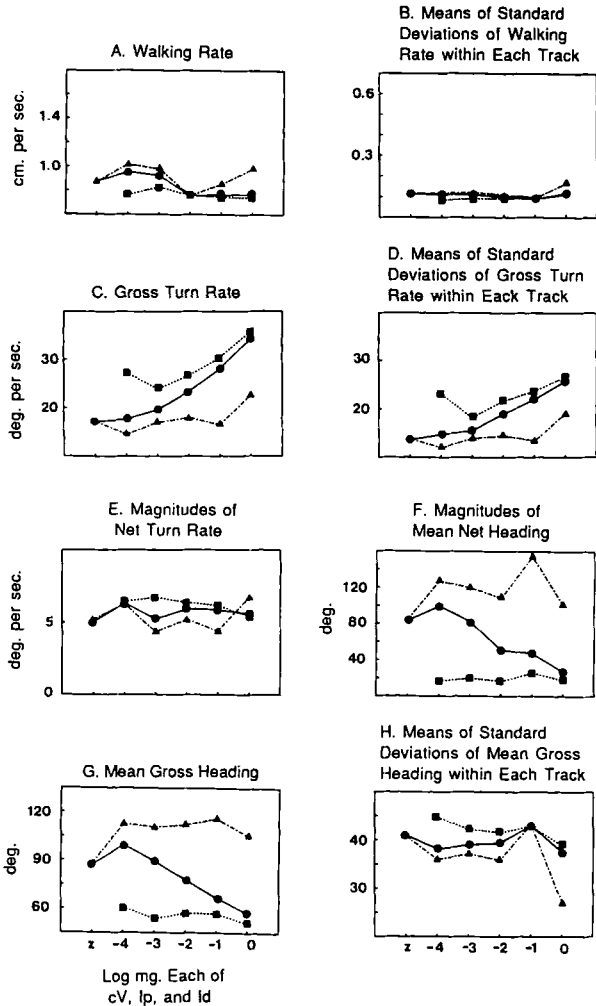


FIG. 6. Comparison of behavioral measurements for beetles that did and did not reach the source of pheromone in the experiment with the 60-min diffusion period. Solid circles indicate response of whole population; squares indicate response of beetles that reached source; and triangles indicate response of beetles that did not reach source.

increased with dosage among those beetles that reached the source (Table 2: dosage, rs; Figure 6C). The threshold for these effects appeared to be at about 10^{-3} – 10^{-1} mg (Figures 5 and 6), while the proportion of beetles reaching the source seemed to have a lower threshold, at most 10^{-4} mg (Figure 4).

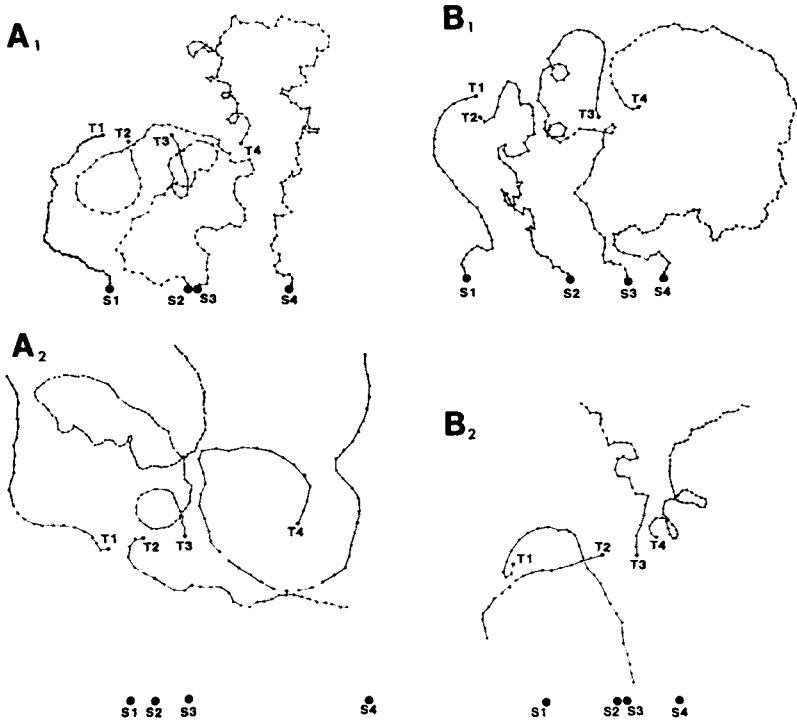


FIG. 7. Randomly selected examples of trails from two treatments in the dosage series experiment with the 60-min diffusion period, illustrating the dissimilarity between the behavior of beetles that did and did not reach the source, and the similarity between the behavior of beetles that reached sources of dissimilar dosage. A = 10^{-1} mg treatment; B = 10^{-4} mg treatment; A₁, B₁ = beetles that reached the source of pheromone; A₂, B₂ = beetles that did not reach the source of pheromone; T_x = start of trail x; S_x = location of source for trail x. Points on tracks show location of beetles at 1-sec intervals and are enlarged for clarity. Distance between release points of beetles and source was 16–18 cm.

DISCUSSION

Since more than one beetle could be tested in a single arena, the chemotaxis assay allowed the testing of large numbers of insects, which was important to later experiments. It also created environmental conditions appropriate for eliciting the desired orientation response. Without a source of pheromone, the beetles oriented at random within the observation chamber. Orientation was definitely nonrandom when pheromone was present in the assay (Figure 7). A

beetle, wandering in a random straight line away from its point of release, had a probability of approximately 0.021 of contacting the pheromone source by chance, given the dimensions of the pheromone dispenser and arena. As dosage increased, the proportion of beetles reaching the source increased well above the levels expected by chance (Figure 4).

This change in the proportion of beetles reaching the source could have been brought about by a change in the directional vector (Bell and Tobin, 1982) of orientation of every beetle in a treatment, or a change in the proportion of beetles entering into orientation, or a combination of the two. At first glance the summaries of heading made over entire treatments made the first hypothesis appear adequate. When the second hypothesis occurred to us, the problems with the hypothesis of increasing accuracy became apparent. The decrease in turn radius as dosage increased meant that beetles in higher dosages had trails that were less linear than those of beetles in lower dosages, although earlier studies had suggested that they might have been expected to be more linear. Then an examination of samples of trails suggested that the behavior of beetles that did and did not reach the source differed from one another (Figure 7). This was confirmed by the analysis of beetles that did and did not reach the source, which showed that not only was the behavior of responding beetles qualitatively different from that of nonresponding beetles, but also that dosage had small effects on the behavior of beetles within either of the two groups (Table 2, Figures 5 and 6). In particular, increasing dosage did not decrease the mean heading of beetles that reached the source. Beetles within this group all seemed to be about equally accurate in approaching the source, despite differences in dosage. These results indicated that the major factor in pheromone source location was whether or not a dosage elicited orientation behavior. A beetle's reaching the source was a good indication that it was responding to the pheromone and entering into such behavior. In addition, since the behavior of beetles that did not reach the source was so dissimilar to that of beetles that reached the source, not only in heading but in walking and turning rate as well, most of this group was probably not responding to the pheromone at all.

There are two main exceptions to this pattern. Dosage had a significant, although small, effect on the gross turning rate of beetles that reached the source, at least in the experiment with the 60-min diffusion period (Table 2: dosage, rs, 60 min; Figure 6C). Second, neither dose nor group had any significant effect on the magnitude of the net turning rate (Table 2, Figures 5E, 6E). In other words, the paths of all beetles tended to be about equally straight overall. On the other hand, not only their directional vector (Bell and Tobin, 1982) but their walking and turning rates depended strongly on whether they reached the source.

Since the behavior of a female nearing a source was similar at all dosages, and since higher dosages should produce steeper gradients, the behavior of a

beetle nearing a source of pheromone also might not vary much with distance from the source, even if the concentration changed rapidly and perhaps nonlinearly. A direct test of this hypothesis is possible in the 60-min experiment in the treatment with a 1-mg dose of pheromone, in which the concentration of pheromone in the air had been quantified. The concentration changed rapidly with distance from the source (Figure 2). In contrast, the gross turning rate changed slightly between the source and the release point of the beetles (Figure 8). The mean gross heading also changed very little as the beetles neared the source, except for an apparent decrease within the last few centimeters of the source (Figure 9).

On the other hand, the beetles walked distinctly more slowly as they moved farther from their release point (Figure 10). This result had not been expected, since dosage had little effect on the walking rate. A similar response might have been expected if the beetles were tiring, recovering from an escape response, or otherwise acclimating to their surroundings. However, the walking rate did not appear to depend on distance from the source or from the release point of

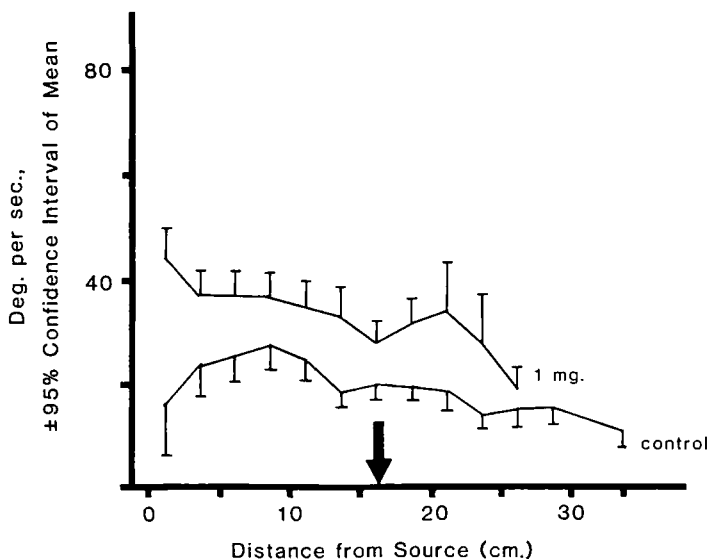


FIG. 8. The relationship between the gross turning rate and the distance to the source of pheromone, in the experiment with the 60-min diffusion period. The curve for the 1-mg treatment is based on data from only those beetles ($N = 23$) that reached the source, while the curve for the control treatment is based on data from all the beetles in the treatment ($N = 24$). Arrow marks the approximate release point of the beetles.

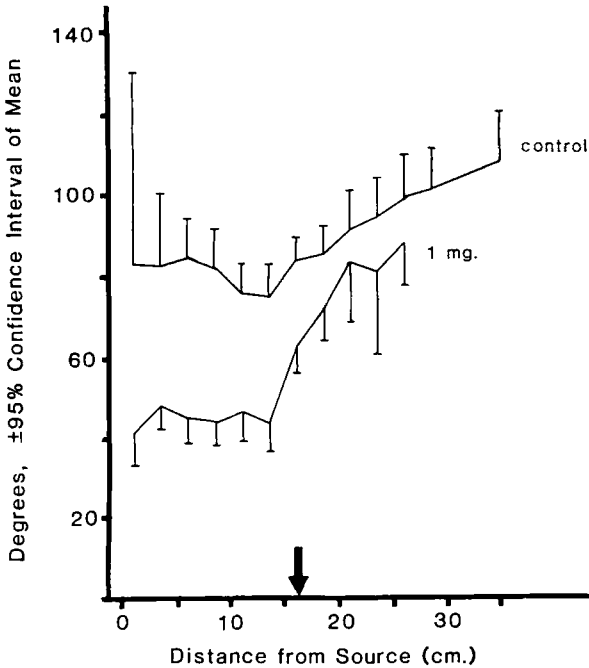


FIG. 9. The relationship between the mean gross heading and the distance to the source of pheromone, in the experiment with the 60-min diffusion period. The curve for the 1-mg treatment is based on data from only those beetles ($N = 23$) that reached the source, while the curve for the control treatment is based on data from all the beetles in the treatment ($N = 24$). Arrow marks the approximate release point of the beetles.

the beetles in the control treatment, although such a decline has been noted in other instances in blank arenas. Thus, the decrease in walking rate may be an effect of the pheromone, but perhaps only in part.

Similar analyses of responses vs. distance to the source at other dosages and of the 30-min diffusion period showed similar results, but (1) the variability of the mean gross headings was often larger at lower dosages; (2) the decrease in gross heading was often more gradual and less steplike, especially in the 30-min experiment; and (3) the gross turning rate was more variable, especially in the 30-min experiment (Akers, 1985). These results suggest that concentration or change in concentration modulated the behavioral responses to a small extent, once a beetle responded to the pheromone. Higher concentration and/or ascending a gradient caused a slight increase in the gross turning rate (Figure 8) (Akers, 1985), a possible decrease in the walking rate (Figure 10) (Akers, 1985), and

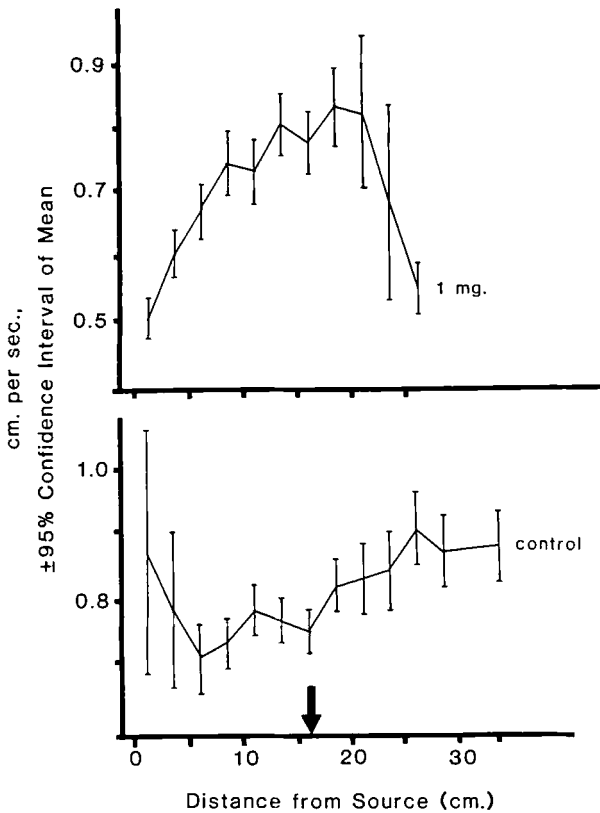


FIG. 10. The relationship between the walking rate and the distance to the source of pheromone, in the experiment with the 60-min diffusion period. The curve for the 1-mg treatment is based on data from only those beetles ($N = 23$) that reached the source, while the curve for the control treatment is based on data from all the beetles in the treatment ($N = 24$). Arrow marks the approximate release point of the beetles.

the heading decreased gradually as the source was neared, except for a more abrupt decrease that usually occurred within 0–5 cm of the source (Akers, 1985, 1989). However, dosage exerted its major effect by altering the probability that orientation behavior was elicited, rather than affecting the linearity and heading of every beetle exposed to the source.

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MULTICOMPONENT ATTRACTANT FOR FEMALE SCREWORM FLIES, *Cochliomyia hominivorax*, IN BOVINE BLOOD¹

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Abstract—An olfactometer bioassay was used to follow attractant for screwworm flies, *Cochliomyia hominivorax*, in steam distillates of bovine blood under different distillation and storage conditions and after HPLC separation of components in a water-methanol gradient. In addition, fly responsiveness was examined in relation to sex and ovarian stage. Gravid and vitellogenic nullipars were attracted to the blood, although the former predominated four to one. Males did not respond at a dose that attracted 76% of gravid females. Maximum attractiveness occurred when distillate was stored in sealed glass ampoules. An argon atmosphere made storage at ambient temperatures feasible, but offered no advantage during storage at ca. -60°C or during distillation. The HPLC separation produced four fractions that duplicated the attractiveness of the distillate when recombined but showed little activity when presented as two-fraction, and most three-fraction, mixtures. Availability of the HPLC fractions for combination with other samples will facilitate location via bioassay of attractant components in samples obtained from subsequent or alternate isolations that preserve only one or two elements of the multicomponent mixture.

Key Words—Screwworm fly, *Cochliomyia hominivorax*, Diptera, Calliphoridae, attractant, host finding.

¹Mention of a proprietary product does not constitute an endorsement or recommendation for its use by the USDA.

INTRODUCTION

The screwworm fly, *Cochliomyia hominivorax* (Coquerel), is a pest of livestock throughout the tropical and subtropical Americas, although populations have been eradicated north of the Isthmus of Tehuantepec in Mexico via sterile insect releases (Krafsur et al., 1987). Females oviposit on newborn or wounded animals (Knipling, 1979) and can use wound-derived olfactory cues in host seeking (Hammack and Holt, 1983). They are attracted by odors from a variety of materials simulating hosts, including decomposing meat and liver (Bishopp, 1937; Jones et al., 1976), incubated blood (DeVaney et al., 1973), and spent artificial diet from larval rearing (Adams et al., 1979). Gram-negative bacteria associated with screwworms also produce attractive odors, and microbial contamination of the above hostlike materials is probably necessary for their activity because neither sterile blood nor fresh wounds are attractive (DeVaney et al., 1973; Eddy et al., 1975; Bromel et al., 1983; Hammack and Holt, 1983; Hammack et al., 1987). These attractants are of value to monitor and perhaps suppress screwworm populations (Knipling, 1979; Snow et al., 1982), but little headway has been made in their chemical isolation and identification. Progress has been hampered by a number of factors including variable effectiveness of different batches of attractant (DeVaney et al., 1973), batch variation in dose-response patterns (Hammack, unpublished data), and loss of biological activity with attractant fractionation (Grabbe and Turner, 1973).

Difficulties due to batch variation can be minimized by isolation of attractant from a dried bovine blood that is available in quantity and stable when crude to heat treatment and frozen storage (Hammack and Holt, 1983). In this paper, we (1) report on the responsiveness of screwworm flies to the dried-blood attractant in relation to sex and ovarian status, (2) describe methods to conserve attractiveness of a steam distillate of the blood during storage, (3) describe a distillate fractionation procedure that yielded nearly full recovery of biological activity after recombination of four fractions that individually showed little attractiveness, and (4) illustrate how availability of these four fractions will facilitate bioassay of samples that preserve only one or two components of the total attractive mixture.

METHODS AND MATERIALS

Insects. Screwworms of the V-81 strain derived from multiple egg masses collected in Veracruz, Mexico, were used in generations 28-51, except that sex comparisons were done in generation 91. Larvae were reared on a beef-based diet, and adults maintained on a 12 : 12 light-dark photoperiod at $25 \pm 1^\circ\text{C}$

and $50 \pm 10\%$ relative humidity with access to corn syrup and water (Hammack, 1984). Anesthesia (CO_2) was used only to separate the sexes.

Chromatographic fractions and stored distillates were bioassayed with 10- to 15-day-old nulliparous females that had been held with males through 6 days of age. Such females should be gravid and more than 90% inseminated (Hammack et al., 1987); this was confirmed by dissecting 25 females per rearing group when each group was first sampled for bioassay. We used the ovarian scoring scheme of Adams and Reinecke (1979) in which egg stage 10 indicates the presence of mature eggs and stages 4-8 the presence of vitellogenic terminal oocytes. Females with sperm in their spermathecae were considered mated.

Similar procedures were used to obtain flies for the sex and ovarian status tests, except that cages of males were also set up following sexing at six days and females with vitellogenic-stage ovaries were assayed three to four days after emergence. The latter females had been held with males from emergence and sexed on the day of testing without anesthesia. Their ovarian stage and mating status were determined by dissection after assay.

Attractant. A single batch of spray-dried bovine blood (Rath Packing Co.), which had been stored dry for up to eight years at ca. -20°C , was used in all experiments. A crude 10% by weight mixture of the dried blood in water (concentration ca. 50% that of whole blood) was assayed in tests done to examine fly responsiveness in relation to sex and ovarian status, as well as in the distillate stability tests where it served as a bioassay standard. Some aliquots were stored at ca. -20°C up to 96 days after reconstitution, which does not affect attractiveness (Hammack and Holt, 1983).

A steam distillate to be fractionated chromatographically was prepared in air from 200 g of the dried blood in 1 liter of distilled water. Steam was passed through the stirred mixture at $\leq 100^\circ\text{C}$, and then condensed and frozen in a flask cooled by dry ice. The first 500 ml were thawed and divided into 10-ml aliquots that were stored in glass screwtop vials at $\leq -60^\circ\text{C}$ in the dark.

Two additional batches of steam distillate, one prepared in air and the other in argon, were used to examine the cause of rapid activity losses encountered when distillate was handled at room temperature and to determine the effect of oxygen exclusion during distillation and sample storage. In both air and argon runs, 135 ml of distillate were collected over a 60- to 75-min period in an ice-chilled receiver from a mixture of 45 g of the dried blood, 600 ml of distilled water, and three drops of methyl silicone oil (to reduce foaming). Portions (8-ml) of each distillate, held ice-cold, were sealed in glass ampoules or placed in glass screwtop vials. The ampoules holding distillate prepared under argon were filled and sealed in an argon atmosphere. The samples were stored one to two months in the dark at $\leq -60^\circ\text{C}$ or ambient temperature (ca. $20-25^\circ\text{C}$) and then tested for attractiveness.

Distillate aliquots (4-ml) were chromatographed by preparative high-pressure liquid chromatography (HPLC) on a C₈ Radial Pak Cartridge (10.0 × 0.8 cm, Waters Associates, Inc.) employing a water-methanol solvent at a flow rate of 1.5 ml/min. The water-methanol ratio was changed during the first 25 min from 98 : 2 to 10 : 90 via a linear program and then held constant. Five major fractions eluting over a 36-min period were initially collected for use in bioassays; three of these were subdivided in subsequent separations (Figure 1). Controls for each fraction consisted of solvent blanks collected after water was injected onto the column.

A 10-ml aliquot of distillate was applied to a column (8.0 × 0.8 cm) that had been packed with C₁₈ Bondapak (10- μ l, Waters Associates, Inc.) and equilibrated with distilled water. The column was eluted with methanol (MeOH), and the first 1.0 ml after the void volume, hereafter referred to as the MeOH eluate, collected for bioassay.

Behavioral Bioassays. Bioassays were conducted at $25 \pm 1^\circ\text{C}$ as previously detailed (Hammack and Holt, 1983) in olfactometers that each consisted of a vertical tube with four choice ports located upwind (Adams et al., 1979). Samples were placed in two diagonally positioned ports (treated ports), while the other two held only water and served as controls. The fluid volume per port was adjusted with water to 1.0 ml, except for several instances when sample volumes exceeded 1.0 ml. Twenty-five females were routinely tested per olfactometer; they were discarded after one use.

In tracing attractant in the HPLC fractions of the blood distillate, we first established that the recombined mixture of all fractions was attractive and then determined which fractions could be eliminated from the mixture without loss of activity. This approach was used to avoid discarding fractions that might contribute to attractiveness of the mixture but show little activity when bioassayed individually. The isolated fractions were recombined for bioassay in the proportions in which they were originally present in the steam distillate, except when otherwise indicated.

The MeOH eluate was bioassayed alone and in combination with HPLC fraction 1, which contained the more water-soluble constituents of the blood distillate.

Calculations and Statistics. Test doses are given as sample volumes and as relative strengths (RS), the latter to facilitate comparisons among different combinations of fractions of varying volume. The attractant activity in a 75- μ l dose of steam distillate was arbitrarily assigned an RS of 1, and the other dosages expressed accordingly after assuming 100% recovery of attractant activity after fractionation.

The equation for the percentage of females attracted was: $[(T - C)/N] \times 100$, where T is the total number of flies trapped in treated ports, C is the total number trapped in control ports, and N is the total number tested. Because

of variation in responsiveness to the attractant (Hammack, 1984), statistical comparisons were limited to treatments tested simultaneously with matched insects, except for one case involving the MeOH eluate in which the response to an internal standard remained constant between assays done at different times and another in which gravid and vitellogenic females were assayed but not reared simultaneously. Treatments within tests were compared with respect to the total number of flies in treated ports, control ports, and the rest of the olfactometer using χ^2 calculated from contingency tables. Further comparisons within tests of more than two treatments were made only when overall χ^2 values were significant ($P \leq 0.05$). Independence of these comparisons was maintained when applicable by successive pooling of similar data sets as described by Steel and Torrie (1960).

RESULTS AND DISCUSSION

Gravid and vitellogenic nullipars both responded to the dried-blood attractant; however, the gravid ones predominated nearly four to one (Table 1). The vitellogenic flies were 70% inseminated and ranged in ovarian stage from 4–8, with a mean of 6.8 ± 1.1 (\pm SD). Too few males reacted to demonstrate significant attraction, although the test dose was sufficient to attract 76% of similarly aged gravid females (Table 1).

Wounded host animals likewise attract both gravid and vitellogenic nullipars, which feed and oviposit at the wound, but few males (Guillot et al., 1977, 1978). Females also predominate in response to steam distillate prepared from cultures of the bacterium, *Providencia rettgeri* (Hammack et al., 1987), although attraction of vitellogenic nullipars was only just detectable. Thus, with

TABLE 1. RELATIONSHIP OF SEX AND OVARIAN STATUS TO RESPONSIVENESS OF SCREWORM FLIES TO 10% BY WEIGHT DRIED BOVINE BLOOD IN WATER

Test	Status	Dose (μ l)	N	No. trapped ^a		Attracted (%) ^b
				T	C	
1	Gravid female	300	100	77	1	76 a
	Male	300	100	19	11	8 b
2	Gravid female	120	200	169	8	81 a
	Vitellogenic female	120	151	37	4	22 b

^aTotal number trapped in treated (T) and control (C) olfactometer ports.

^bResponses within tests followed by different letters are significantly different at $P < 0.001$ by contingency χ^2 .

respect to the three population segments examined here, the dried blood showed a response profile similar to that characterizing hosts and bacterial attractants.

The following results with the steam distillates of blood prepared and stored under various conditions implicated both volatility and oxidative degradation in distillate lability at ambient temperatures, but showed no advantage to air exclusion during distillation or low-temperature storage. Distillate prepared in air or argon attracted significantly more females by 23% (Table 2, test 1) or 36% (data not shown), respectively, when it was stored at $\leq -60^{\circ}\text{C}$ in a sealed ampoule as compared with a screwtop vial. Samples stored in ampoules at ambient temperature were less attractive than those held frozen only when ampoules contained air, not when they contained argon (Table 2, tests 2-5). However, when held frozen in ampoules, distillate prepared and stored in air did not differ in attractiveness from that similarly handled in an argon atmosphere (Table 2, test 6).

Responses of gravid females to the HPLC fractions of the steam distillate

TABLE 2. RESPONSES OF GRAVID SCREWORM FLIES TO BLOOD AND STEAM DISTILLATES (SD) OF BLOOD PREPARED AND STORED IN AIR OR ARGON AND STORED IN DARKNESS AT AMBIENT TEMPERATURE OR $\leq -60^{\circ}\text{C}$ IN SEALED AMPOULES OR SCREWTOP VIALS FOR 1-2 MONTHS ($N = 100$)

Test	Test sample	Dose (μl)	No. trapped ^a		Attracted (%) ^b
			T	C	
1	Ampoule, frozen air SD	10	82	2	80 a
	Vial, frozen air SD	10	61	4	57 b
2	Frozen, air ampoule SD	20	64	5	59 a
	Ambient, air ampoule SD	20	28	12	16 b
3	Frozen, argon ampoule SD	20	63	9	54 a
	Ambient, argon ampoule SD	20	72	5	67 a
4	Blood standard ^c	10	52	1	51 a
	Air, ambient ampoule SD	10	18	1	17 b
5	Blood standard ^c	10	56	5	51 a
	Argon, ambient ampoule SD	10	81	3	78 b
	Argon, ambient ampoule SD	5	75	7	68
	Argon, ambient ampoule SD	2.5	50	5	45
6	Air, frozen ampoule SD	8	68	1	67 a
	Argon, frozen ampoule SD	8	67	4	63 a

^aTotal number trapped in treated (T) and control (C) olfactometer ports.

^bResponses within tests followed by the same letter are not significantly different at $P \leq 0.05$ by contingency χ^2 .

^cTen percent by weight dried bovine blood in water.

of blood are summarized in Table 3. The recombined mixture of all HPLC fractions (fractions 1–5, Figure 1) attracted fewer gravid females than the original distillate, but this was due to interference from the MeOH solvent rather than any loss of attractant activity during fractionation (Table 3, tests 1–3). Fractions 2, 4G, and 5 could be eliminated from the mixture without affecting fly responses (Table 3, test 4). Fractions 3A, 3E, 3F and 4D, 4E, 4F were also deleted on the basis of preliminary data; their failure to contribute to attractancy was confirmed only later (Table 3, tests 10, 11, and 15). Their elimination along with fractions 2, 4G, and 5 reduced the amount of MeOH in test samples to levels unlikely to interfere with attraction (Table 3, test 5).

The deletion of fraction 1, eluting between 3 and 9 min and containing the more polar components, or the removal of portions of fraction 1 consisting of either 1A or 1B resulted in a significant decline in attractancy of the mixture (Table 3, tests 5 and 6). The magnitude of the decline ranged from 50% to 60% of females attracted when either 1A or 1B was deleted, and this was higher than the 20–25% reduction expected even if doses of the crude distillate were halved (Table 3, test 1). This discrepancy suggests that at least some of the active fractions acted synergistically or else affected different behaviors contributing to the overall attraction response.

Neither fractions 3B–D nor 4A–C could be deleted from the mixture without a significant loss of attractiveness (Table 3, test 6). Activity in fraction 4A–C was traced to the 4A area (Table 3, test 7), but not 4B (test 8) or 4C (test 16). Activity in fraction 3B–D was associated with 3C (Table 3, tests 12 and 13), but not 3B or 3D (tests 14 and 15).

Mixtures of the HPLC fractions that contained fractions 1A, 1B, 3C, and 4A were generally as attractive as the original steam distillate (Table 3, tests 10, 11, 12, and 13); any discrepancies were usually slight (test 9) or attributable to the methanol solvent (test 3). Therefore, we concluded that attractiveness of the steam distillate could be accounted for by components present in these four fractions.

The percentage of females attracted to the MeOH eluate alone failed to exceed 17% and showed little dose dependence over a 50-fold range from 1.5 to 75 μ l (Table 4, test 1). However, responses as high as 55% and dose dependence were demonstrable when 1.5–3 μ l were tested in combination with HPLC fraction 1, which also showed little or no attractancy when tested by itself (Table 4, tests 2 and 3). The MeOH eluate was able to substitute for HPLC fractions 3 and 4 and did so at doses consistent with concentration of the less polar attractant components by as much as 10 times in the MeOH eluate as compared with the steam distillate (Table 4, test 4).

Although some of the HPLC fractions may prove unsuitable for further isolation, their availability for mixing with samples generated in subsequent or

TABLE 3. RESPONSES OF GRAVID SCREWORM FLIES TO STEAM DISTILLATE (SD) OF BLOOD AND TO HPLC FRACTIONS OF DISTILLATE

Test	Test sample ^a	Dose			No. trapped ^c		Attracted (%) ^d
		μ l	RS ^b	N	T	C	
1	SD	75	1.0	100	72	8	64 ^c
	SD 50%	75	0.5	100	50	7	43
	SD 25%	75	0.25	100	31	14	17
2	Fractions 1-5	1087	1.0	300	98	27	24 a
	SD in blanks ^f 1-5	1087	1.0	300	111	28	28 a
3	SD	75	1.0	135	118	2	86 a
	SD in blanks 1-5	1087	1.0	125	49	14	28 b
4	1,2,3,4A-F,4G,5	1012	1.0	300	96	35	20 a
	1,3,4A-F,blanks 2,4G,5	1012	1.0	300	104	38	22 a
5	SD	75	1.0	100	73	5	68 a
	1A,1B,3B-D,4A-C	324	1.0	100	82	4	78 a
	1A,3B-D,4A-C	240	1.0	100	37	10	27 b ^b
	1B,3B-D,4A-C	240	1.0	100	34	13	21 b ^b
6	1,3B-D,4A-C	161	0.5	100	81	1	80 a
	1,3B-D	113	0.5	100	57	7	50 b
	1,4A-C	133	0.5	100	30	6	24 c
	3B-D,4A-C	78	0.5	100	14	3	11 d
7	1,3B-D,4A	52	0.2	100	56	7	49 a
	1,3B-D	46	0.2	100	17	5	12 b
8	1,3B-F,4A,4B,4C	42	0.1	300	122	22	33 a
	1,3B-F,4A,4C,blank 4B	42	0.1	300	109	15	31 a
9	SD	15	0.2	300	173	28	48 a ^a
	SD in blanks 1,3B-F,4A,4C	92	0.2	300	174	16	53 a ^a
	1,3B-F,4A,4C	92	0.2	300	144	15	43 b
10	SD	15	0.2	300	143	13	43 a
	1,3B-D,3E,3F,4A,4C	76	0.2	300	132	12	40 a
	1,3B-D,3F,4A,4C,blank 3E	76	0.2	300	155	12	48 a
11	SD	15	0.2	300	148	21	42 a
	1,3B-D,3F,4A,4C	76	0.2	300	174	23	50 a
	1,3B-D,4A,4C,blank 3F	76	0.2	300	164	18	49 a
12	SD	30	0.4	100	75	4	71 a ^a
	1,3B,3C-D,4A,4C	124	0.4	100	68	6	62 a ^a
	1,3B,4A,4C,blank 3C-D	124	0.4	100	36	8	28 b
13	SD	15	0.2	100	74	8	66 a ^a
	1,3B,3C,3D,4A,4C	62	0.2	100	81	4	77 a ^a
	1,3B,3D,4A,4C,blank 3C	62	0.2	100	44	9	35 b
14	1,3B-C,3D,4A,4C	31	0.1	300	172	27	48 a
	1,3B-C,4A,4C,blank 3D	31	0.1	300	170	30	47 a
15	1,3A-B,3C,4A,4C,4D-G	46	0.1	300	133	30	34 a
	1,3C,4A,4C,blanks 3A-B,4D-G	46	0.1	300	135	26	36 a

TABLE 3. Continued

Test	Test sample ^a	Dose		N	No. trapped ^c		Attracted (%) ^d
		μl	RS ^b		T	C	
16	1,3C,4A,4C	54	0.2	200	83	32	26 a
	1,3C,4A,blank 4C	54	0.2	200	79	30	25 a

^aHPLC fractions, designated by number and further subdivided by letter, are defined in Figure 1.

^bRelative strength, see text for explanation.

^cTotal number trapped in treated (T) and control (C) olfactometer ports.

^dResponses within tests followed by the same letter are not significantly different at $P \leq 0.05$ by contingency χ^2 .

Brackets indicate responses that were pooled before further comparisons were made among samples within tests.

^e $Y = 78.1 (\log 100X) - 247.4$, $r^2 = 0.996$, $P < 0.05$, $Y = \% \text{ attracted}$, $X = \text{dose}$ (expressed as % of full strength SD).

^fSolvent controls for indicated fractions.

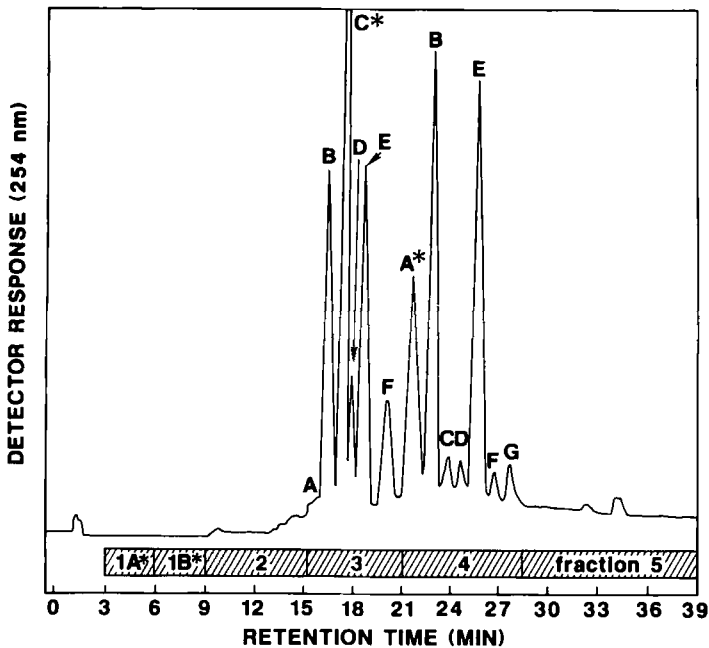


FIG 1. Representative HPLC chromatogram of a steam distillate of bovine blood. The numbers 1-5 and lettered subdivisions of 1, 3, and 4 designate fractions that were tested in bioassays. Asterisks indicate fractions showing attractant activity.

TABLE 4. RESPONSES OF GRAVID SCREWORM FLIES TO MeOH ELUATE (E) OF BLOOD STEAM DISTILLATE FROM BONDAPAK COLUMN TESTED ALONE AND IN COMBINATION WITH HPLC FRACTION 1 (F1)

Test	Test sample	Dose		N	No. trapped ^b		Attracted (%) ^c
		μ l	RS ^a		T	C	
1	E	1.5	0.2	100	23	13	10 ac
	E	15.0	2.0	100	36	22	14 b
	E	75.0	10.0	100	33	16	17 ba
	MeOH solvent	75.0		100	16	23	-7 c
	Distillate	15.0	0.2	200	140	16	62
2	E	6.0	0.8	100	29	20	9 a
	F1	134.0	0.8	100	30	4	26 b
	F1 (67 μ l) + E (3 μ l)	70.0	0.4	100	66	11	55 c
3	Distillate	30.0	0.4	100	73	8	65 c
	F1	34.0	0.2	100	21	12	9 a
	F1 (34 μ l) + E (0.15 μ l)	34.2	0.02 ^d	100	27	10	17 a
	F1 (34 μ l) + E (1.5 μ l)	35.5	0.2	100	65	10	55 b
4	F1 (17 μ l) + E (0.75 μ l)	17.8	0.1	125	74	14	48 a
	F1 (17 μ l) + F3,F4 (13 μ l)	30.0	0.1 ^e	125	69	16	42 a
	F1 (17 μ l) + F3,F4 (1.3 μ l)	18.3	0.01 ^e	125	49	16	26 b

^aRelative strength, see text for explanation.

^bTotal number trapped in treated (T) and control (C) olfactometer ports.

^cResponses within tests followed by the same letter are not significantly different at $P \leq 0.05$ by contingency χ^2 . Brackets indicate similar responses that were pooled before further comparisons were made among samples within tests.

^dHPLC fraction 1 at RS 0.2 mixed with MeOH eluate at RS 0.02.

^eHPLC fraction 1 at RS 0.1 mixed with HPLC fractions 3B, 3C, 4A, 4C at RS 0.1 or 0.01.

alternate steps (e.g., the MeOH elution of the Bondapak column) can facilitate identification via bioassay of samples containing only one or two components of the attractant mixture. Because the four fractions recombined were about as attractive as the original steam distillate, the relative volumes of the fractions provide a guideline for mixing the attractant constituents in proportions expected to yield strong and dose-dependent responses.

Odors from screwworm-infested wounds were attractive to gravid screwworm flies in earlier olfactometer tests (Hammack and Holt, 1983), but a plateau occurred with increasing dose at a response level well below the maximum obtainable with the dried-blood attractant. These differing dose-response patterns could indicate that attractants from the blood and wound are chemically and functionally distinct; however, many batches of *P. retzgeri* attractant also elicit a dose-response plateau at 20–40% of females attracted (Hammack and

Bromel, unpublished data), even though some produced by the same bacterial isolate consistently evoke strong and dose-dependent responses (Bromel et al., 1983; Hammack et al., 1987). Such variation in dose-response patterns could alternatively arise simply from quantitative or qualitative variation among components of one multicomponent system; for example, peak responses to sex pheromones of the lightbrown apple moth are limited at ratios of components that deviate significantly from those released by the female (Bellas and Bartell, 1983).

Results of the present study, including demonstration of the multicomponent nature of the blood attractant, the apparent synergism among active fractions, and the similarity in the segments of the population responding to dried-blood and host attractants, are consistent with the hypothesis that blood and hosts share a common multicomponent attractant. That attractiveness of host-associated materials apparently depends upon microbial contamination provides even stronger support (DeVaney et al., 1973; Eddy et al., 1975; Bromel et al., 1983). Thus, procedures developed for chemical isolation of the blood attractant may prove applicable to host and defined bacterial sources that have been difficult to examine because of limited quantities or variable compositions.

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ELECTROANTENNOGRAM RESPONSES OF *Campoletis sonorensis* (HYMENOPTERA: ICHNEUMONIDAE) TO CHEMICALS IN COTTON (*Gossypium hirsutum* L.)

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Abstract—Combined gas chromatography–electroantennogram (GC-EAG) recording of *Campoletis sonorensis* (Cameron) responses to cotton plant volatile chemicals was performed. *C. sonorensis* antennal olfactory receptors respond differentially to green leaf, mono-, and sesquiterpene chemicals that have been identified previously in cotton. EAG depolarizations to green leaf chemicals were greater than to terpenes.

Key Words—*Campoletis sonorensis*, parasitoid, Hymenoptera, Ichneumonidae, cotton, *Gossypium hirsutum*, host habitat location, green leaf chemical, monoterpene, sesquiterpene, electroantennogram, olfaction, volatile.

INTRODUCTION

Parasitoid host-finding behavior includes the steps of habitat preference, potential host community location, and host location (Vinson, 1984). While proceeding through this hierarchy of behavioral events, parasitoid sensory systems are bombarded by environmental stimuli, some of which are more important in eliciting behavioral responses than others.

Plants and their chemicals are important in parasitoid habitat location (Vinson, 1981). *Diaeretiella rapae* responds to allyl isothiocyanate as a cue to locate its host on crucifers (Read et al., 1970). Similarly, *Heydenia unica* uses α -pinene as a cue to locate its host on pine trees (Camors and Payne, 1972). Elzen et al. (1983, 1984) described short-range orientation and attraction of *Campoletis sonorensis* to host-free cotton and to some volatile cotton chemicals

and subsequently (1986) demonstrated the long-range orientation and attraction of *C. sonorensis* to host-free cotton in a wind tunnel bioassay.

Parasitoid host habitat location in these cases involves perception of volatile chemicals. The sensory cells that detect volatile, low molecular weight organic molecules are olfactory receptor neurons usually located on the antennae (Schneider, 1964). The electroantennogram (EAG) (Schneider, 1957) records cumulative antennal olfactory receptor responses to volatile chemicals. Visser (1983) suggested that herbivores show differential olfactory sensitivities depending on the volatile chemistry of their host plant. It would be adaptively advantageous for parasitoids to evolve similar receptors for plant chemicals and utilize them in host habitat location. While Visser (1983) stressed the importance of green leaf chemicals (short-chain alcohols and aldehydes that smell like grass; Visser et al., 1979) in habitat location by herbivores, he realized that there are many other volatile plant chemicals that influence leaf odor and perhaps insect behavior. A similar approach must be taken to decipher host habitat location by insect parasitoids. The objective of this study was to use combined gas chromatography-electroantennogram (GC-EAG) studies (Arm et al., 1975) to record cumulative olfactory receptor responses of *C. sonorensis* to volatile chemicals that have been previously identified in cotton plants.

METHODS AND MATERIALS

Insects. *C. sonorensis* were reared on a culture of *Heliothis virescens* larvae, which were fed an artificial diet described by Vanderzant et al. (1962). Adult parasitoids were mated and maintained in 300-ml screened cages and fed honey-distilled water (1:2) on cotton pads. Cages were held in an environmental chamber at $26 \pm 2^\circ\text{C}$ with a photoperiod of 16:8 hr (light-dark).

Chemical Stimuli. Chemicals used for olfactory stimuli are listed in Table 1. These chemicals were chosen based on their presence in cotton essential oil (Minyard et al., 1967; Hedin et al., 1971; Elzen et al., 1984), their volatility as defined by Amoore (1982), their attraction to insects of various species (Camors and Payne, 1972; Visser and Ave, 1978), and their behavioral effects described for *C. sonorensis* (Elzen et al., 1984). All chemicals were diluted to desired concentration with hexane (pesticide grade, Fisher Scientific).

Combined Gas Chromatography-Electroantennogram Methodology. The GC-EAG technique used was similar to that previously described by Struble and Arm (1984). Chemicals were injected in 1.0- μl aliquots via an on-column injector (Scientific Glass Engineering, Inc.) to a 0.22-mm ID \times 25-m vitreous silica BP1 capillary column in a Varian 3700 GC equipped with a flame ionization detector (FID). Upstream from the FID, makeup gas was added at 20 ml/min and effluent was then split 5:1 (EAG-FID) by a fixed outlet splitter

TABLE 1. SOURCE AND PURITY OF ODOROUS STIMULI USED IN EAG STUDIES.

Compound	Chemical purity (%)	Source
Green Leaf		
Hexanal	99	Aldrich
<i>cis</i> -3-Hexen-1-ol	98	Aldrich
<i>trans</i> -2-Hexen-1-ol	97	Aldrich
<i>trans</i> -2-Hexenal	99	Aldrich
Heptanal	95	Aldrich
Monoterpenes		
α -Pinene	>99	Aldrich
β -Pinene	>98	Aldrich
Myrcene	85	Aldrich
Limonene	97	Aldrich
Sesquiterpenes		
β -Caryophellene	>94	International Flavors and Fragrances
α -Humulene	>96	Fluka
β -Caryophellene oxide	>98	Givaudan
Gossonorol	>98	Synthesized

system (Williams and Vinson, 1980). EAG routed effluent passed through a 200°C exit port into a modified water cooled (24°C) condenser (1 cm ID). The distance between effluent introduction into the condenser and antenna preparation was 20 cm. Purified air, humidified by bubbling through distilled water at 300 ml/min, swept the effluent over the antenna preparation.

EAGs were recorded using Ag-AgCl electrodes encased in glass capillaries filled with 0.75% NaCl. Heads of mated 6-day postemergent female *C. sonorensis* were excised, and the antenna tip was inserted into the recording electrode. The indifferent electrode was inserted into the mandibles. EAG signal was amplified 100-fold by a DC preamplifier (George Johnson Electronics) and viewed on a Tektronix 561B oscilloscope. EAG and FID signals were simultaneously recorded for later analysis on Hewlett Packard 3390A integrators.

Dilutions of heptanal (10^1 - 10^{-2} μg) were administered to a series of antenna preparations. All EAG responses were measured relative to a 1.0- μg heptanal standard. Each presentation of stimuli was performed as an individual GC injection and was followed by the application of a standard. Green leaf chemicals were presented in 1.0- μg doses while the mono- and sesquiterpenes were tested at 1.0- and 10.0- μg doses. Test chemicals were applied in a sequence within each of the three chemical groups with each sequence being applied to a different antenna preparation. Prior to testing data for significance, a regression of mean and variance was performed to determine if a pattern in the data was present that required a data transformation (Ott, 1984). The mean percent

response relative to the standard was tested for all chemicals at all doses using Duncan's multiple range test ($P < 0.05$) (Duncan, 1955).

RESULTS AND DISCUSSION

A typical GC-EAG response is shown in Figure 1. The mean response of female *C. sonorensis* to the 1.0- μg heptanal standard was -1.02 mV (SE ± 0.14 , $N = 10$). Precision of flame ionization and antenna detection time is evident. The reliability of the EAG was tested by dose response (Figure 2). EAG responses to each dose of heptanal were significantly different by Duncan's multiple range test ($P < 0.05$).

Response to Green Leaf Chemicals. Green leaf volatile chemicals elicited larger *C. sonorensis* EAG responses than the mono- and sesquiterpenes tested, with heptanal producing the strongest response (Figure 3A). Six-carbon alcohols and aldehydes elicited EAG responses that were significantly smaller than heptanal.

Differing olfactory responses to green leaf volatile chemicals are not surprising. Similar results have been shown for several orders of insect (Dickens, 1984; Dickens and Boldt, 1985; Dickens et al., 1986; Guerin and Visser, 1980;

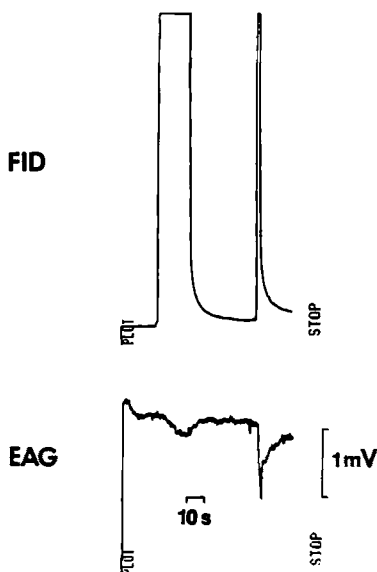


FIG. 1. Typical combined GC-EAG responses of female *C. sonorensis* to 1.0 μg of heptanal.

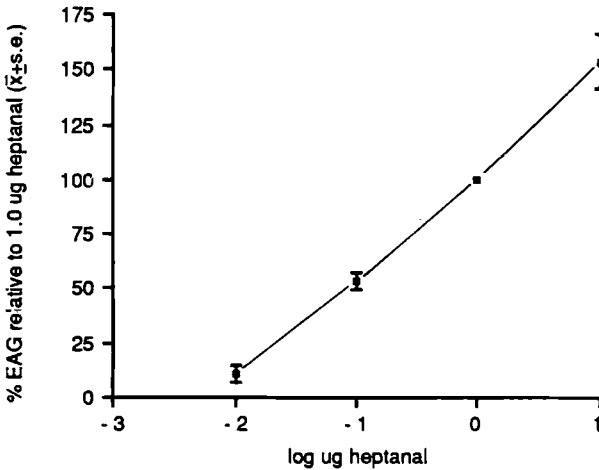


FIG. 2. Mean EAG response of female *C. sonorensis* to dilutions of heptanal.

Van der Pers, 1981; Visser, 1979). Although Dickens et al. (1986) have demonstrated this phenomenon in Hymenoptera, and Lecomte and Pouzat (1985) have recorded parasitic Hymenoptera EAG responses to plant chemicals, this is the first suggestion of different olfactory response to similar green leaf chemicals in parasitic Hymenoptera.

Response to Monoterpenes. Monoterpenes elicited significantly smaller EAG responses than most green leaf chemicals and smaller, but not significantly smaller, responses than sesquiterpenes. α -Pinene was the only monoterpene that stimulated *C. sonorensis* at a 1.0- μ g dose (Figure 3B). *C. sonorensis* responded electrophysiologically to 10.0 μ g of α -pinene, myrcene, and limonene, while β -pinene never elicited any response at either dose. Responses to 10.0 μ g of α -pinene and limonene were significantly greater than to the other monoterpenes tested. These data suggest that *C. sonorensis* is capable of discriminating differences not only in green leaf molecular structure but also in monoterpene structure, based on differences in EAG response to α -pinene and β -pinene.

Response to Sesquiterpenes. *C. sonorensis* EAG responses to sesquiterpenes were similar to responses elicited by monoterpenes in that doses of 10.0 μ g were needed to approach parity of responses elicited by 1.0 μ g of green leaf chemicals (Figure 3C). All the sesquiterpenes tested except gossonorol produced small EAG responses at a 1.0- μ g dose. *C. sonorensis* responded to all the sesquiterpenes tested at 10.0 μ g. β -Caryophellene oxide elicited the strongest responses at both 1.0 and 10.0 μ g.

While it is important that EAG data and data from behavioral studies are compared, it must be stressed that EAG responses do not necessarily reflect

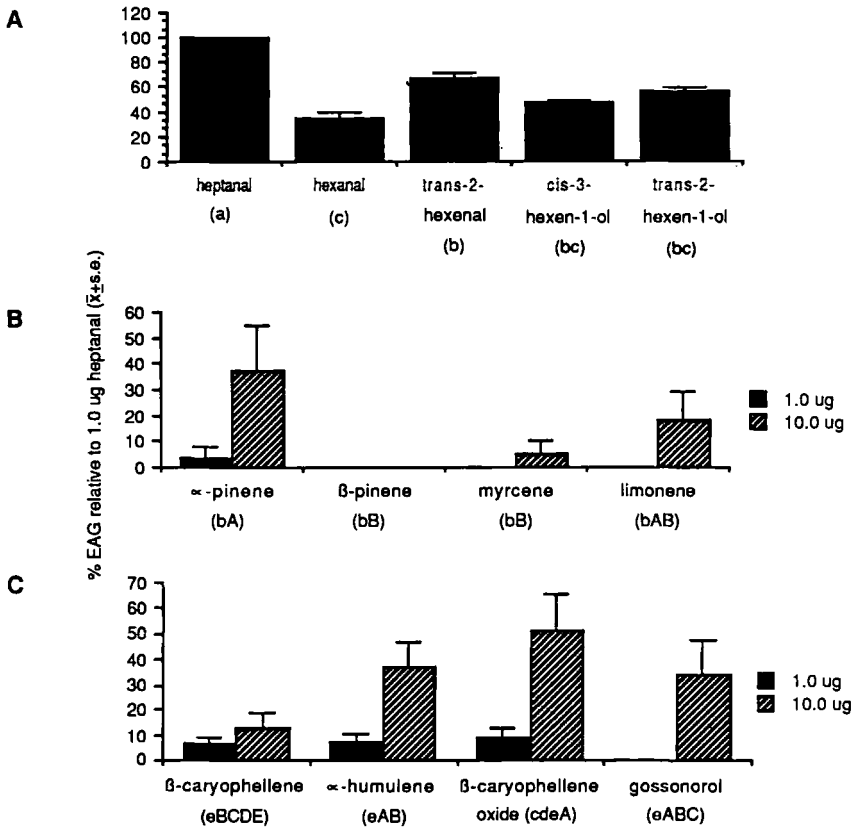


FIG. 3. Mean EAG response of female *C. sonorensis* to 1.0- μ g doses of green leaf chemicals (A), 1.0- and 10.0- μ g doses of monoterpenes (B), and 1.0- and 10.0- μ g doses of sesquiterpenes (C). Chemicals followed by the same letter are not significantly different with capital letters representing 10.0- μ g doses, Duncan's multiple range test ($P < 0.05$, $N = 10$).

central integration of peripheral stimuli and therefore do not indicate the resulting behavioral response. When electrophysiology data described here are compared to *C. sonorensis* behavioral Y-tube bioassay data of Elzen et al. (1984), minor differences are evident. The strongest EAG response to sesquiterpenes was toward β -caryophellene oxide, while behavioral data indicate a greater attraction to gossonorol. Behaviorally *C. sonorensis* did not respond to β -caryophellene, which was the least responsive sesquiterpene tested electrophysiologically. Behavior and EAG data appear to agree well with respect to α -humulene and β -caryophellene oxide responses.

CONCLUSION

Little research has been conducted on insect parasitoid attraction to specific plant chemicals and chemical blends other than those previously mentioned of Camors and Payne (1972), Elzen et al. (1984), and Read et al. (1970). While electrophysiological techniques have been utilized as tools in insect herbivore olfactory-mediated behavior studies, to date few electrophysiological studies have been conducted on parasitoids. Such research may elucidate the mechanisms involved in perception of behavioral stimuli by organisms used for biological control of insects.

The most striking similarity between our data and those of EAG studies with insect herbivores (Guerin and Visser, 1980; Kozłowski and Visser, 1981; Visser, 1979) is that substantially larger doses of terpenes than of green leaf chemicals are required to elicit responses near parity. While such data may be a function of volatility or chemical plating in the odor delivery tube, it may also suggest that molecules with smaller dose olfactory sensitivities (green leaf chemicals) could be more important in longer distance orientation to an odor source while chemicals with larger dose olfactory sensitivities (terpenes) may be more important at shorter ranges. Evidence supporting this supposition has been provided by studies comparing glanded and glandless cotton varieties, which differ mainly in production of terpenes (Elzen et al., 1985). *C. sonorensis* preferentially landed on glanded (high terpene) cotton if a choice were allowed, but landed on glandless (low terpene) cotton in no-choice wind tunnel studies (Elzen et al., 1986). Future behavioral studies may clarify such questions.

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DIHYDROPYRROLIZINE ATTRACTANTS FOR ARCTIID MOTHS THAT VISIT PLANTS CONTAINING PYRROLIZIDINE ALKALOIDS

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Abstract—Adults of three species of arctiid moths (*Cisseps fulvicollis*, *Ctenucha virginica*, and *Halysidota tessellaris*) are attracted to plants that contain pyrrolizidine alkaloids (PAs). The moths use olfactory cues to locate these plants, then feed on leaves, flowers, and roots with the proboscis. To investigate the chemical basis of attraction, sticky traps were baited with roots of a PA-containing plant, *Eupatorium maculatum*, alkaloids of *E. maculatum*, and several derivatives of these alkaloids. Volatile derivatives of the bicyclic pyrrolizidine skeleton attracted all three arctiid species. The dihydropyrrolizines, (*S*)-(+)-hydroxydanaidal and (*R*)-(–)-hydroxydanaidal, proved to be the most attractive compounds tested, accounting for over 70% of the moths captured. Different alkaloid derivatives attracted different proportions of male and female *Cisseps*. Both (*S*)-(+)-hydroxydanaidal (52% male) and (*R*)-(–)-hydroxydanaidal (71% male) attracted a significantly lower percentage of *Cisseps* males than *E. maculatum* roots (87% male). *Cisseps* males possess eversible scent organs (coremata) that are displayed during courtship. Analysis of coremata extracts revealed the presence of hydroxydanaidal. *Cisseps* moths thus resemble danaine and ithomiine butterflies, both in their attraction to PA sources and in the presence of PA derivatives in the male scent organs.

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Key Words—*Cisseps fulvicollis*, *Ctenucha virginica*, *Halysidota tessellaris*, hydroxydanaidal, pyrrolizidine alkaloids, *Eupatorium maculatum*, Lepidoptera, Arctiidae, Ctenuchinae, coremata, male pheromones, attractants.

INTRODUCTION

Adults of many species of butterflies and moths visit damaged or decaying plants that contain pyrrolizidine alkaloids (PAs) (Edgar et al., 1973, and references cited; Pliske, 1975a; Goss, 1979; Boppré, 1981, 1986; Brown, 1984b). These Lepidoptera feed on the surface of the plants, often by regurgitating fluid, then reimbibing the presumably enriched solution. Visitation is restricted largely to members of four subfamilies: Danainae and Ithomiinae of the Nymphalidae, and Arctiinae and Ctenuchinae of the Arctiidae. Within each species, visitation may be further limited to one sex. While males typically predominate at baits of PA-containing plants, female biased and unbiased visitation patterns occur in some species.

Studies of both nymphalids and arctiids have documented that adults imbibe and store alkaloids while feeding at PA sources (Edgar et al., 1976a, 1979; Edgar, 1982; Brown 1984a, b; Dussourd, 1986). These alkaloids may be subsequently used as a defense against predators (Eisner, 1980; Brown, 1984a, b), as precursors for male courtship pheromones (references cited in Ackery and Vane-Wright, 1984, and in Boppré, 1984), and/or as a "nuptial gift" whereby the male contributes alkaloids to the female during mating (Brown, 1984a, b; Dussourd, 1986; Dussourd et al., 1988).

In this report we consider the chemical basis of attraction of Lepidoptera to PA-containing plants. Previous studies documented that attraction is mediated by olfactory cues (Beebe, 1955; Edgar et al., 1973), and specifically by volatile derivatives of plant alkaloids (Pliske, 1975a; Pliske et al., 1976). Ithomiine butterflies locate PA sources by detecting "esterifying acids" (such as viridifloric acid, Figure 1F) or breakdown products of these acids (Pliske, 1975a; Pliske et al., 1976). Esterifying acids presumably dissociate from the amino-alcohol nucleus of pyrrolizidine alkaloids when plant tissues decay. Unlike ithomiine butterflies, arctiid moths appear to respond to derivatives of the amino-alcohol nucleus. Pliske (1975a) and Pliske et al. (1976) were able to attract a small number of moths with amino-alcohol derivatives (such as retronecine, Figure 1D), but failed to attract any moths with esterifying acids.

Our study arose from the serendipitous discovery that volatiles from the scent organs (coremata) of male *Pyrrharctia isabella* (J.E. Smith) (Arctiidae) attract adults of three other arctiid species. Sticky traps baited with excised coremata or coremata extracts of *P. isabella* males captured the following moths: *Cisseps fulvicollis* Hubner (five males, one female), *Ctenucha virginica*

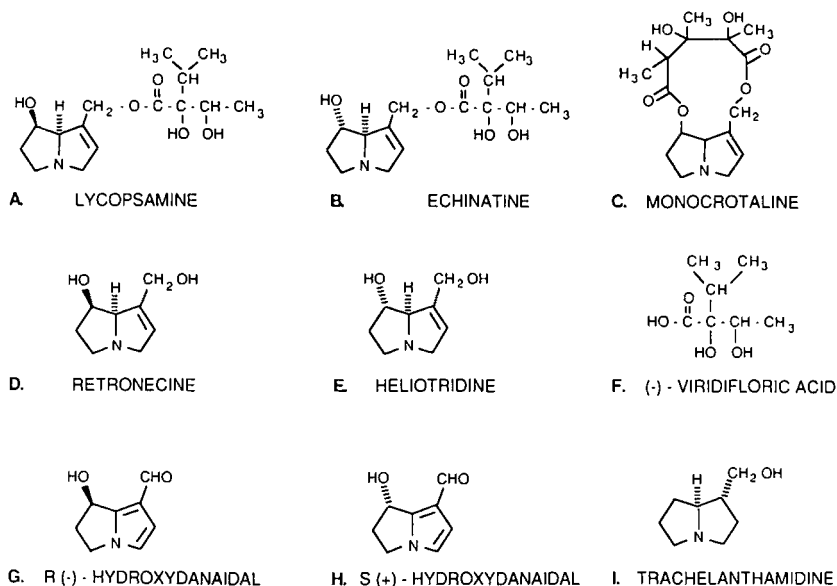


FIG. 1. Structures of pyrrolizidine alkaloids and derivatives.

Esp. (20 females, one male), and *Halysidota tessellaris* J.E. Smith (three females). Moreover, we observed a *Cisseps fulvicollis* male feeding upon excised *P. isabella* coremata, and a *Halysidota tessellaris* female feeding on a filter paper disk impregnated with an extract of *P. isabella* coremata (Figure 2).

Dussourd (1986) had collected over 800 individuals of the same three arctiid species at baits of two PA-containing plants, *Eupatorium maculatum* L. and *E. capillifolium* Lam. (Asteraceae). The proportion of males and females attracted to the *Eupatorium* baits and *Pyrrharctia* coremata was similar for each arctiid species. These results suggested that *Eupatorium* and *Pyrrharctia* might contain similar attractants. A dihydropyrrolizine, hydroxydanaidal (Figure 1G, H), had been identified previously from the coremata of several other arctiid species that feed on alkaloid-containing plants as larvae (Culvenor and Edgar, 1972; Conner et al., 1981; Schneider et al., 1982). We hypothesized that hydroxydanaidal might also be present in *P. isabella* coremata (subsequently documented by Krasnoff et al., 1987); that it was responsible for attracting *Cisseps fulvicollis*, *Ctenucha virginica*, and *Halysidota tessellaris* to the core-matal extracts; and that this compound, or some closely related amino-alcohol derivative(s), mediated the attraction of these species to *Eupatorium* plants.

Herein we describe a trapping study in which we compare the attraction of arctiid moths to a natural plant lure, *E. maculatum*, alkaloids from *E. macu-*

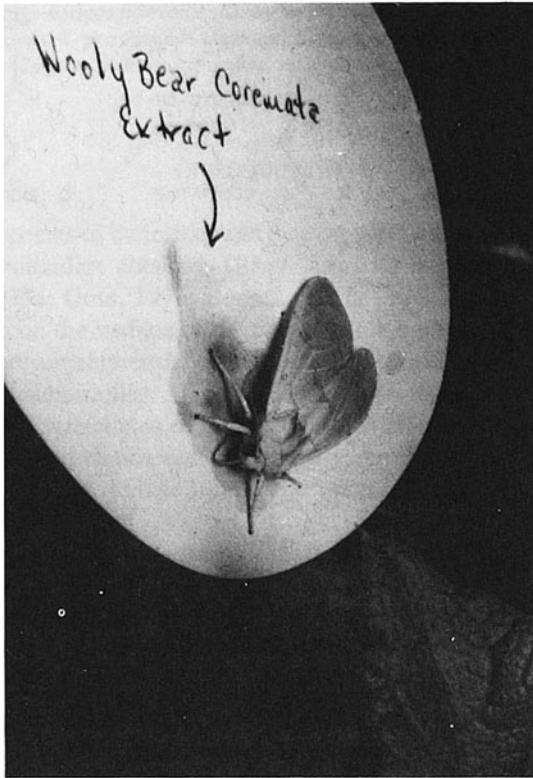


FIG. 2. *Halysidota tessellaris* female feeding in the field on a filter paper disk impregnated with a hexane extract of *Pyrrharctia isabella* coremata.

latum, and several derivatives of these alkaloids including hydroxydanaidal. We also document the presence of hydroxydanaidal in *Cisseps fulvicollis* coremata. While dihydropyrrolizines such as hydroxydanaidal have been identified from the male scent organs of numerous danaine visitors to PA plants (references in Ackery and Vane-Wright, 1984), there has been no parallel evidence for the presence of pyrrolizidine derivatives in the scent organs of male arctiid visitors.

METHODS AND MATERIALS

Attractancy of Pyrrolizidine Alkaloids and Derivatives. Pherocon 1C sticky traps were baited with the following treatments: (1) 10 g (fresh weight) *Eupatorium maculatum* roots; (2a) two diastereomeric alkaloids from *E. maculatum*, lycopsamine (Figure 1A) and echinatine (Figure 1B), tested in 1982 in the same

3:2 ratio as they occur in *E. maculatum* roots (J. Resch, S. Goldstein, and J. Meinwald, unpublished); (2b) monocrotaline (Figure 1C) (tested in 1983); (3) retronecine (Figure 1D), a hydrolysis product of lycopsamine; (4) heliotridine (Figure 1E), a hydrolysis product of echinatine; (5) (*R*)-(-)-hydroxydanaidal (Figure 1G), an oxidation product of retronecine; (6) (*S*)-(+)-hydroxydanaidal (Figure 1H), an oxidation product of heliotridine; (7) (2*S*, 3*S*)-(-)-viridifloric acid (Figure 1F), the acid produced by hydrolysis of either echinatine or lycopsamine; and (8) a control consisting of 25 μ l dichloromethane. Compounds were dissolved in either dichloromethane (treatments 2a, 5, 6) or methanol (2b, 3, 4, 7) at a concentration of 50 μ g/25 μ l, and tested at a dosage of 50 μ g/trap. The *E. maculatum* roots served as a standard for determining the activity of synthetic compounds relative to this natural attractant. We tested excised roots since arctiids primarily visit the roots of uprooted *E. maculatum* plants in preference to other plant parts (Dussourd, 1986).

Fresh *E. maculatum* roots were bundled with thread and placed on the sticky surface in the center of traps. Each of the other treatments and the control were prepared by pipetting 25 μ l solution into the open end of a rubber septum (5 \times 9 mm) similarly placed in the center of the traps. Traps were suspended ca. 2 m off the ground, approximately 15 m apart, along a fence in a grassy field in Tompkins County, New York. Treatments and trap liners were replaced and the trap positions rerandomized every seven days.

Daily counts were taken of the number of arctiid moths in each trap from August 17 to September 17, 1982, and from June 24 to July 30, 1983. Moths were removed and sexed every other day in 1982 and daily in 1983. Weekly totals were transformed to $\log_{10}(x + 1)$ and submitted to analysis of variance. Differences between treatment means were determined using Waller and Duncan's (1969, 1972) BSD rule. For the 1982 test, differences in the sex ratio of moths captured were determined by a *z* test using the normal approximation of the binomial (Snedecor and Cochran, 1967) with Sidak's inequality to adjust alpha levels for > 1 pairwise comparison (Sidak, 1967).

Chemistry of Cisseps Coremata Secretion. *Cisseps fulvicollis* males were collected at lights in Manatee County, Florida, during April 1984, and in Ontario County, New York, during August and September 1985. The males were either frozen and stored at -20°C for later use or were dissected alive within two days of capture. Coremata were extruded by gently squeezing the abdomen. They were then gripped with forceps, excised with a razor blade, and placed in redistilled dichloromethane (ca. 150 μ l/male) to which hexadecane had been added as an internal standard. The coremata were allowed to soak in solvent for at least 1 hr at 20°C before the extract was analyzed by GLC.

Capillary GLC was conducted with either a 25-m cross-linked methyl silicone column (ID 0.31 mm) or a 5% phenylmethyl silicone column (ID 0.20 mm), programmed from 80 to 200°C at $10^{\circ}/\text{min}$ after an initial hold of 1 min,

or with a 30-m Supelcowax 10 column (ID 0.25 mm) programmed from 80 to 230° at 15°/min after an initial hold of 1 min. Quantification of hydroxydanaidal was achieved on the 5% phenylmethyl silicone column by comparing the electronically integrated peak areas of hydroxydanaidal and its degradation products (Krasnoff et al., 1987) with the peak area of a known amount of internal standard (hexadecane). Electron impact (EI) mass spectra were obtained with a Hewlett-Packard 5985 GC-MS system using a methyl silicone capillary column.

A bioassay used in the identification of the male pheromones of *Phragmatobia fuliginosa* L. and *Pyrrharctia isabella* (Krasnoff et al., 1987) was also utilized as a probe for small amounts of dihydropyrrolizines. This assay, hereinafter referred to as the *Pyrrharctia* bioassay, employs the wing-fluttering and clicking response of *Pyrrharctia* females to courting males (or pheromone samples) as evidence for the presence of hydroxydanaidal (the male pheromone) or related compounds.

RESULTS

Attractancy of Alkaloids and Derivatives. In the 1982 tests, only a single arctiid species, *Cisseps fulvicollis*, was captured in the sticky traps. The dihydropyrrolizine, hydroxydanaidal, proved to be a potent attractant for *Cisseps* adults. Of the 977 *Cisseps* collected, 53% were captured in the (*S*)-(+)-hydroxydanaidal traps (Table 1), with an additional 19% in the (*R*)-(-)-hydroxydanaidal traps. The remaining test compounds were either moderately active (heliotridine), weakly active (retronecine), or inactive (viridifloric acid, lycopsamine, and echinatine). The relative attractancy of compounds [(*S*)-(+)-

TABLE 1. ATTRACTION OF *Cisseps fulvicollis* TO PYRROLIZIDINE ALKALOIDS AND RELATED COMPOUNDS

Treatment	Moths/week ^a
(<i>S</i>)-(+)-hydroxydanaidal	129.75 a
(<i>R</i>)-(-)-hydroxydanaidal	46.75 b
<i>Eupatorium maculatum</i> roots	43.50 bc
Heliotridine	19.25 c
Retronecine	4.25 d
Dichloromethane	0.50 e
(-)-Viridifloric acid	0.25 e
Lycopsamine/echinatine	0.25 e

^aMean number of *Cisseps* captured per week (*N* = 4 weeks). Means followed by the same letter are not significantly different at the 0.05 level (Waller and Duncan's BSD rule).

hydroxydanaidal > (*R*)-(-)-hydroxydanaidal > heliotridine > retronecine] remained constant from week to week. Analysis of the daily capture totals indicated that all attractive compounds remained active for the duration of each week, and none of the unattractive compounds became activated with time.

The sex ratios of *Cisseps* in traps differed greatly among treatments (Figure 3). The strong male bias (87% male) of the roots treatment agreed with the 89% male bias reported by Dussourd (1986) for moths collected at uprooted *Eupatorium* plants, but differed significantly from both the (*S*)-(+)-hydroxydanaidal (52% male) and (*R*)-(-)-hydroxydanaidal (71% male) treatments. The per-

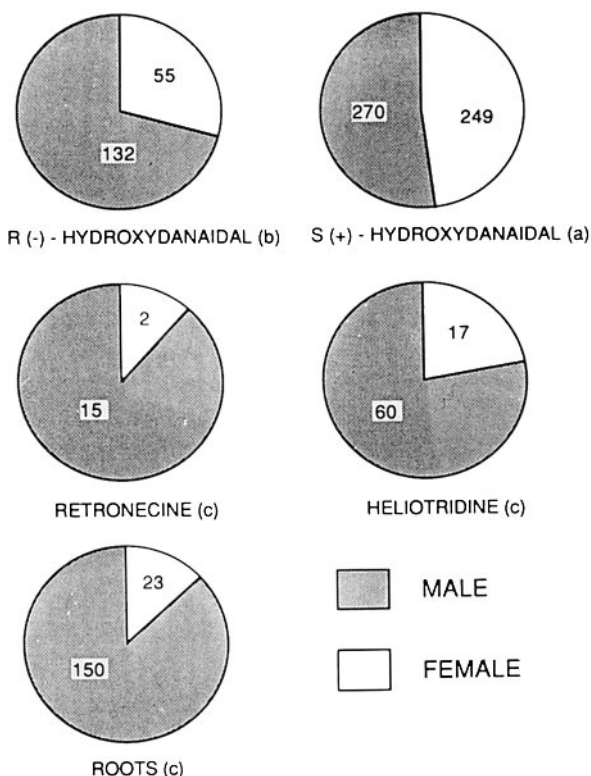


FIG. 3. Sex of *Cisseps fulvicollis* adults captured in sticky traps baited with either pyrrolizidine alkaloids, related compounds, or roots of an alkaloid-containing plant, *Eupatorium maculatum*. Numbers represent the total capture of each sex in the 1982 test. Treatments followed by the same letter do not differ in sex ratio of captured moths at the 0.05 level by a z test using the normal approximation of the binomial (Snedecor and Cochran, 1967) with Sidak's inequality to adjust alpha levels for > 1 pairwise comparison (Sidak, 1967).

centage of *Cisseps* males captured in the (*S*)-(+)-hydroxydanaidal traps varied between weeks (range 31–64%); nevertheless, (*S*)-(+)-hydroxydanaidal consistently attracted a lower proportion of males than any of the other treatments.

The 1983 test, conducted during the flight periods of *Cisseps*, *Ctenucha*, and *Halysidota*, documented the general attractancy of hydroxydanaidal to arctiids that visit plants containing pyrrolizidine alkaloids (Table 2). Of the 172 moths captured, 69% were found in the (*S*)-(+)-hydroxydanaidal traps, with an additional 20% in the (*R*)-(–)-hydroxydanaidal traps. As previously noted for the 1982 tests, moths attracted to the (*S*)-(+)-hydroxydanaidal traps exhibited less skewed sex ratios (*Cisseps*, 66% male; *Ctenucha*, 28%; *Halysidota*, 31%) in comparison with the strong sex biases reported by Dussourd (1986) for arctiids visiting *Eupatorium* baits (*Cisseps*, 89% male; *Ctenucha*, 19%; and *Halysidota*, 4%).

Corematal Chemistry. Artificially extruded coremata of *Cisseps fulvicollis* males elicited a positive response in the *Pyrrharctia* bioassay, indicating the presence of hydroxydanaidal or related compounds. A compound present in *Cisseps* corematal extracts was identified as hydroxydanaidal by comparing its retention times on polar and nonpolar GLC columns and its EI mass spectrum with retention times and spectra of an authentic sample of hydroxydanaidal. Individual *Cisseps* males were found to contain small, but variable, amounts of hydroxydanaidal in their coremata (mean = 31 ng/male; range: 2–122 ng) (Figure 4).

TABLE 2. ATTRACTION OF ARCTIID MOTHS TO PYRROLIZIDINE ALKALOIDS AND RELATED COMPOUNDS

Treatment	<i>Cisseps</i> ^a		<i>Ctenucha</i> ^a		<i>Halysidota</i> ^a		Total ^b
	F	M	F	M	F	M	
(<i>S</i>)-(+)-hydroxydanaidal	12	23	23	9	35	16	23.6 a
(<i>R</i>)-(–)-hydroxydanaidal	1	2	20	6	4	2	7.0 b
<i>E. maculatum</i> roots	0	2	0	2	6	3	2.6 c
Heliotridine	0	1	1	0	1	0	0.6 d
Retronecine	0	1	0	2	0	0	0.6 d
Dichloromethane	0	0	0	0	0	0	0.0 d
(–)-Viridifloric acid	0	0	0	0	0	0	0.0 d
Monocrotaline	0	0	0	0	0	0	0.0 d

^aTotal number of moths of each sex (F = female, M = male) captured during the entire study.

^bMean number of moths (three species pooled) captured per week (*N* = 5); means followed by the same letter are not significantly different at the 0.05 level (Waller and Duncan's BSD rule).

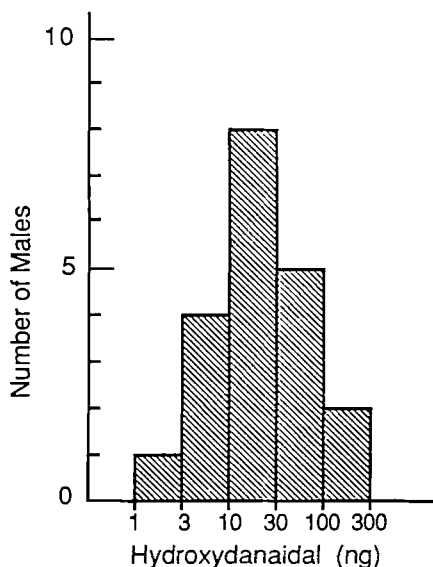


FIG. 4. Frequency distribution of hydroxydanaidal titers in the coremata of *Cisseps fulvicollis* males ($N = 20$) collected in Ontario County, New York. Bars represent the number of males within the indicated range.

DISCUSSION

Attractancy of Alkaloids and Derivatives. Our results suggest that arctiid moths locate plants containing pyrrolizidine alkaloids by detecting volatile derivatives of the amino-alcohol nucleus. We captured a total of 959 moths in traps containing amino-alcohols [retronecine, heliotridine, (*S*)-(+)- and (*R*)-(-)-hydroxydanaidal], but collected only two moths in traps baited with monoesters (lycopsamine/echinatine), a macrocyclic diester (monocrotaline), and an esterifying acid (viridifloric acid).

Pliske (1975a) also attracted arctiid moths with amino alcohols and related compounds (one moth each at retronecine, laburnine, isoretronecinol, and 1-methylene-pyrrolizidine). Another amino alcohol (heliotridine), a dihydropyrrolizine (1-formyl-6,7-dihydro-5H-pyrrolizine = "danaidal"), and seven esterifying acids were not observed to attract any moths. A small number of moths visited monoesters (10 moths) and diesters (two moths); however, breakdown products in these samples may have been responsible for their activity (Pliske, 1975a; also see Pliske et al., 1976).

To determine the attractiveness of chemicals, Pliske (1975a) impregnated

blotter pads with 1–10 mg of synthetic compounds (20–200 times the amount that we used). It is surprising that he did not collect more moths, especially with heliotridine and retronecine, which we found to be moderately attractive, and with danaidal, which resembles the highly attractive enantiomers of hydroxydanaidal.

While our results suggest that volatile amino-alcohol derivatives attract arctiid moths, other insects evidently respond to different compounds. Edgar et al. (1973) tested a series of dihydropyrrolizines (including hydroxydanaidal) for attractiveness to danaines, but failed to attract any butterflies. Ithomiine butterflies likewise failed to visit amino-alcohol baits (Pliske, 1975a) but were attracted to 50-mg samples of esterifying acids (Pliske et al., 1976). Impure samples appeared to be more active than pure samples, suggesting that breakdown products of the acids mediated attraction. Boppré et al. (1984) tested the attractiveness of 50- and 100-mg samples of a monoester, heliotridine, and a mixture of a diester, senecionine, and a monoester, fuchsisenecionine. Both treatments attracted large numbers of a pyrgomorphid grasshopper, *Zonocerus elegans*.

Whether any of these attractants actually occur in the natural habitats of the attracted insects remains to be demonstrated. Edgar et al. (1973) identified hydroxydanaidal in chloroform rinses of moistened *Heliotropium amplexicaule* Vahl (Boraginaceae) from the New Hebrides. In light of our demonstration of the potent attractancy of hydroxydanaidal, it seems quite likely that dihydropyrrolizines, such as hydroxydanaidal, account for the documented attractiveness of several species of *Heliotropium* to arctiids (Pliske, 1975a, and references cited; Goss, 1979; Boppré, 1981). However, we have no evidence that hydroxydanaidal is present in volatiles produced by *Eupatorium maculatum*, the plant employed in our study. On the contrary, preliminary GLC and mass spectral analyses of airborne volatiles and dichloromethane rinses of moistened *E. maculatum* roots did not indicate the presence of either hydroxydanaidal or two related compounds, heliotridine and retronecine. Furthermore, the *Pyrrharctia* assay indicated that hydroxydanaidal was absent from the volatile collections. In addition, our trapping results with *Ciseps* suggest that compounds other than hydroxydanaidal mediate attraction since both (*R*)-(–)- and (*S*)-(+)–hydroxydanaidal attracted significantly different sex ratios of moths than *Eupatorium* roots.

The actual attractant in *E. maculatum* may resemble hydroxydanaidal in structure. Tsuda and Marion (1963) identified an amino alcohol, trachelanthamidine (Figure 1I), in *E. maculatum* roots. This is an unusual PA in that it occurs in the plant as a nonester. As such it is a relatively small molecule (mol wt = 141) that should be volatile enough to be an attractant in this system. While trachelanthamidine was not tested in our trapping study, two stereoisomers, laburnine and isoretronecinol, were screened by Pliske (1975a); each iso-

mer attracted a single female *Halysidota moraniensis* Schaus. In future studies, sticky traps could be used to test the attractancy of trachelanthamidine and to assay volatile collections from *E. maculatum* and other plants for attractive compounds.

A potential problem with sticky traps concerns the effect of trapped insects on trap attractiveness. Hydroxydanaidal released by captured *Cisseps* males, for example, could conceivably attract other moths, and thus affect capture rates and sex ratios. In our study, this effect was probably insignificant since *Cisseps* coremata contain only small amounts of hydroxydanaidal (< 150 ng/male) relative to the quantity of alkaloids tested in our traps (50 µg/trap). In addition, the trapped males did not extrude their coremata and thus probably did not release coremata secretions.

In previous studies, lepidopteran species have been categorized as male-biased, female-biased, or unbiased, based on their visitation patterns to PA-containing plants (e.g., Pliske, 1975a; Goss, 1979). Our data clearly demonstrate that sex ratios depend not only on the species, but also on the chemical composition of the attractant. In addition, the chirality of the attractant can affect overall attractiveness as well as sex ratio. Not only was (*S*)-(+)-hydroxydanaidal more attractive than its antipode, but also heliotridine, the stereospecific reduction product of the (*S*)-(+)-enantiomer, was more attractive than retronecine, the stereospecific reduction product of the (*R*)-(-) form. (*S*)-(+)-hydroxydanaidal also attracted a significantly greater percentage of females than (*R*)-(-)-hydroxydanaidal (48% vs. 29%), as did heliotridine relative to retronecine (22% vs. 12%). Why *Cisseps* sex ratios differ with different attractants remains a mystery; a comparison of the electrophysiological responses of males and females to different attractants may provide insights into the mechanisms involved.

Coremata Chemistry. *Cisseps fulvicollis* adults resemble danaine and ithomiine butterflies, both in their attraction to PA-containing plants and in their release of PA derivatives from male scent organs. Danaine males disseminate hydroxydanaidal and other dihydropyrrolizines from extrusible abdominal hair-pencils (references in Ackery and Vane-Wright, 1984), while ithomiine males release a lactone derived from esterifying acids (Edgar et al., 1976b) from erectile scent scales along the hind wings.

The coremata of several arctiid species that feed on PA-containing plants as larvae also contain dihydropyrrolizines. The quantity of hydroxydanaidal in *Cisseps* coremata (2–122 ng/male) is small in comparison with these species. Substantially larger amounts of dihydropyrrolizines have been isolated from the following arctiids: *Cretonotos* species—up to 400 µg/male (Schneider et al., 1982); *Estigmene acrea* Drury—up to 150 µg/male (Krasnoff, unpublished data); *Phragmatobia fuliginosa*—up to 1.5 µg/male (Krasnoff et al., 1987); and *Pyr-rharcia isabella*—up to 12.3 µg/male (Krasnoff et al., 1987). In addition, large

quantities of dihydropyrrolizines (up to 415 $\mu\text{g}/\text{male}$) have been recorded in danaine hairpencils (Boppré et al., 1978).

Cisseps males presumably synthesize hydroxydanaidal from PAs acquired during adult feeding. However, the presence of PAs in endophytic fungi (references cited in Belesky et al., 1987) that infect grasses that are potential larval food plants suggests that *Cisseps* larvae may also obtain hydroxydanaidal precursors.

During courtship, *Cisseps* males repeatedly evert their paired tubular coremata in the vicinity of calling females (Meyer, 1984). What effect coremata display and the presumed release of hydroxydanaidal have on *Cisseps* females remains unknown. Dihydropyrrolizines released from the coremata of an arctiid moth, *Utetheisa ornatrix* (Conner et al., 1981), and from the hairpencils of a danaine butterfly, *Danaus gilippus* Cram. (Pliske and Eisner, 1969), elicit a receptive posture from females and mediate male courtship success. In other arctiid species, *Pyrrharctia isabella* and *Phragmatobia fuliginosa*, dihydropyrrolizines released during courtship elicit a stereotyped response in females, but do not appear to affect the outcome of courtship (Krasnoff, 1987). *Cisseps* coremata also contain a nonalkaloidal compound, 12-hydroxy-2-tridecanone (Ubik, 1985), the function of which has not been determined.

It is intriguing that the coremata of *Cisseps* males contain hydroxydanaidal, a potent attractant for *Cisseps* adults. Male scent organs in other species also contain attractants. Extracts of danaine hairpencils attract several species of danaines and elicit feeding (Edgar et al., 1973). Similarly, excised scent scales from ithomiine males attract some ithomiines, which feed on the scales for short periods (Pliske, 1975b; Haber, 1978). In addition, females of both *Cisseps fulvicollis* (Krasnoff, personal observation) and *Danaus plexippus* L. (Danainae) (Urquhart, 1960) have been observed to extend the proboscis during courtship, possibly in response to male displays of scent organs.

Thus, in both butterflies and moths, chemicals in male scent organs stimulate attraction and feeding, behaviors also elicited by PA-containing plants. This association, together with the occurrence of volatile PA derivatives in both scent scales (references in Ackery and Vane-Wright, 1984; Edgar et al., 1976b) and plants (Edgar et al., 1973; references in Bull et al., 1968; Culvenor, 1978), suggests that adults may use the same sensory mechanisms in pair-formation and in location of plants containing PAs (as hypothesized for danaine butterflies by Edgar et al., 1974; Boppré, 1978). Further studies of the chemical cues involved in mating and PA visitation may shed light on these relationships and on the evolution of PA visitation and PA courtship pheromones.

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STRUCTURES, ABSOLUTE CONFIGURATIONS, AND SYNTHESSES OF VOLATILE SIGNALS FROM THREE SYMPATRIC ANT-LION SPECIES, *Euroleon nostras*, *Grocus bore*, and *Myrmeleon formicarius* (NEUROPTERA: MYRMELEONTIDAE).

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Abstract—The thoracic gland of the ant-lion *Euroleon nostras* was found to contain nerol oxide (**1a**) and (Z)-6-undecen-2-ol (nostrenol, **3**) while the species *Grocus bore* contained 10-homonerol oxide (**1b**) and nostrenol (**3**). Nerol (**2a**) and 10-homonerol (**2b**) were found in a third species, *Myrmeleon formicarius*. 10-Homonerol, racemic 10-homonerol oxide, and racemic as well as (R)- and (S)-nostrenol were synthesized. The nerol oxide of *E. nostras* and the 10-homonerol oxide of *G. bore* were found to be racemic, while both species contained optically pure (R)-nostrenol (**28**).

Key Words—*Euroleon nostras*, *Grocus bore*, *Myrmeleon formicarius*, Neuroptera, Myrmeleontidae, nerol oxide, nostrenol, (R)- and (S)-(Z)-6-undecen-2-ol, 10-homonerol oxide, nerol, 10-homonerol, chiral shift reagent, chiral GC phase.

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INTRODUCTION

The three ant-lion species, *Euroleon nostras* (Fourc.), *Grocus bore* Tjed., and *Myrmeleon formicarius* L., are sympatric both in time and space in high populations in limited areas on the northern part of Öland, an island in the Baltic in southern Sweden. Their biology indicates pheromone communication as species-specific isolating mechanisms. A morphological study revealed an unusually big, paired, thoracic gland in the males (Elofsson and Löfqvist, 1974). The gland was present in females only as a tiny remnant, which supports the hypothesis of the gland secretion as a species separating pheromone. Two small brushes, which are present only in males at the basal hind margin of the back wings, fit into small pits on the thorax when the wings are folded. The gland secretion is emptied into the pits and is dispersed in the air by the brushes when the males flutter their wings.

The volatile secretions of the glands in males of *E. nostras*, *G. bore*, and *M. formicarius* were analyzed with capillary gas chromatography and gas chromatography-mass spectrometry (Löfqvist and Bergström, 1980). In each species the secretion consisted of only two major components present in about equal proportions. Only traces of minor components were found. One of the components (MS, $m/e = 170$) was present in *E. nostras* and *G. bore*, but it was not identified. In addition, nerol oxide was identified from males of *E. nostras*. The other component in *G. bore* had a mass spectrum very similar to nerol oxide, and it was suggested to be a homonerol oxide. *M. formicarius* contained nerol and an unknown compound (MS, $m/e = 168$).

The intention with this study was to determine the chemical structures, including the stereochemistry, of significant compounds in *E. nostras*, *G. bore*, and *M. formicarius* as a basis for studies of their biological role. Methods for synthesis of the compounds and their isomers have also been developed.

METHODS AND MATERIALS

Insect Material

Larvae of *E. nostras* and *G. bore* were collected in southern Sweden at Byerum in the northern part of Öland, an island in the Baltic. The larvae were bred in the laboratory up to the time of pupation and emergence of imagines according to the procedure of Löfqvist and Bergström (1980).

Preparation of Extracts

The thoracic glands of 3 to 5-day-old males were dissected and immersed into ca. 200 μ l distilled pentane. After concentration to about 100 μ l the extracts

were run on a preparative capillary gas chromatograph (injected volume: 2 μ l, recovery: 80%) equipped with a revolving fraction collector (Wassgren and Bergström, 1984). The analysis was performed using a fused silica column with stationary phase OV-351, 25 m \times 0.35 mm ID, d_f 1 μ m. Thus, the solvent was removed from the volatile compounds, and a separation of the compounds was simultaneously achieved. The neat compounds were then dissolved in chloroform-*d* for analysis by [1 H]NMR.

Analytical Techniques

Gas Chromatography. Gas chromatographic analyses were performed on a Hewlett Packard (HP) 5880 gas chromatograph equipped with an N/P-detector and a flame ionization detector. Fused silica columns were used with stationary phases as follows: Superox FA, 27 m \times 0.5 mm ID, d_f 0.4 μ m; OV-351, 25 m \times 0.35 mm ID, d_f 1.0 μ m; XE-60-(*S*)-valine-(*S*)-2-phenylethylamide, 50 m \times 0.23 mm ID, d_f 0.12 μ m; Ni(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate], 25 m \times 0.25 mm ID; Mn(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate], 35 m \times 0.25 mm ID.

Mass Spectrometry. Finnigan 4021 GC-MS (quadropole) and LKB 2091 GC-MS instruments (magnetic type) were used.

[1 H]NMR Spectrometry. 1 H (200.3 MHz) NMR spectra were measured in CDCl_3 with tetramethylsilane as an internal standard on a Bruker WP200 spectrometer.

Determination of Absolute Configuration.

Lanthanide-Induced Shift (LIS) Studies on Nerol Oxide (*la*). Several chiral shift reagents were investigated in order to resolve nerol oxide. Tris[3-(trifluoromethylhydroxymethylene)-*d*-camphor]europium [$\text{Eu}(\text{TFC})_3$ Stohler Isotope Chemicals] gave no observable shift changes. Tris[*d,d*-dicampholylmethanato]europium [$\text{Eu}(\text{dcm})_3$, Alfa] gave small shift changes but no shift separation of the two enantiomers. However, the third shift reagent tris[3-(heptafluoropropylhydroxymethylene)-*d*-camphor]europium [$\text{Eu}(\text{HFC})_3$] proved to be successful. The quality of the commercial shift reagents varied a lot. Initially Stohler I.C. delivered a sample that gave shift differences of the enantiomers. However, later deliveries proved inactive (and insoluble in chloroform-*d*). Then samples from Ega, Lancaster Synthesis Ltd., and Aldrich were tried with varying success. Sublimation of shift reagent did not improve the result.

The conditions for the LIS studies had to be worked out carefully. In a typical test run $\text{Eu}(\text{HFC})_3$ dissolved in chloroform-*d* was added in portions (0.1 equiv.) to the nerol oxide (7 mg) in chloroform-*d*. The result of each addition was checked by [1 H]NMR, and a lanthanide reagent to substrate ratio (L/S) of 0.5:1 was found to be optimal. Pentane extracts of thoracic glands from 20

males of *E. nostras* were fractionated by preparative capillary gas chromatography to give ca. 200 μg of nerol oxide. A microtube (5/2.5 mm, Wilmad Cat. No 520-1) was used with a sample volume of 100 μl .

Chiral GC Separations. GC separations of the enantiomers of nerol oxide and 10-homonerol oxide were carried out on a fused silica capillary column (35 m \times 0.25 mm ID) coated with Chirametal Mn(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate] in OV-101 (Schurig and Weber, 1981); conditions: isothermal at 65°C, carrier gas N₂ (9 psi), $\bar{\mu}$ = 16 cm/sec. Retention times: (*S*)-neroloxide, 29.6 min; (*R*)-nerol oxide, 30.6 min; 10-homonerol oxide, 59 min and 61.3 min (tentatively assigned to *S* and *R*, respectively). It is interesting to note that only the enantiomers of nerol oxide could be separated on the closely related phase Ni(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate] in SE-54, 25 m \times 0.23 mm ID (Schurig and Weber, 1984); conditions: isothermal at 93°C, carrier gas N₂ (7 psi), $\bar{\mu}$ = 15 cm/sec, split 40/1. Retention times: (*S*)-neroloxide, 12.6 min; (*R*)-neroloxide, 12.4 min.

Separations of the *E/Z* isomers as well as the enantiomers of synthetic (*Z*)- and (*E*)-6-undecen-2-ol were carried out by tandem GC of the isopropyl carbamate derivatives. The first fused silica capillary column (12 m \times 0.22 mm ID, d_f 0.1 μm) was coated with chemically bonded SE-54. The second fused silica capillary column (50 m \times 0.23 mm ID, d_f 0.12 μm) was coated with XE-60-(*S*)-valine-(*S*)-2-phenylethylamide (König, 1982; König et al., 1982). Running conditions were as follows: isothermal at 120°C for 60 min and then programmed to 165°C at a rate of 1°C/min, carrier gas N₂ (22 psi), $\bar{\mu}$ = 12 cm/sec, N/P-detector.

The isopropyl carbamate derivatives of synthetic and natural materials were prepared according to the procedure described by König et al. (1982). After evaporation of the solvent, the residue was dissolved in ca. 50 μl methylene chloride. Then 20 μl isopropyl isocyanate was added followed by heating in an aluminium block at 100°C for 20 min. The solvent was then evaporated and the residue was dissolved in 20 μl methylene chloride; 2 μl of the solution was injected for analysis on a gas chromatograph as described above.

Synthetic Compounds

Column chromatography separations were made on a column that had been dry packed with Merck 60 silica gel, 230–400 mesh. Light petroleum, bp 40–60°C, with increased amounts of ethyl acetate (0, 1.25, 2.5, 5, 10, 20, 40, and 80%) was delivered by a metering pump at a rate of 30 ml/min for 12.5 mm ID columns. Analytical GLC of the synthetic compounds was performed on a Pye Unicam 204 instrument with an FID detector connected to a computing integrator; 25-m columns were used coated either with Carbowax 20 M or with SP-1000. A Finnigan model 4021 spectrometer connected to an INCOS data

system was used to record GC-MS spectra. The [^1H]NMR spectra were recorded in CDCl_3 with tetramethylsilane as internal standard using Varian EM360, Jeol PMX60Si, and Bruker WP200 spectrometers. The coupling constants (J) are in Hz. The following abbreviations are used: DMSO = dimethyl sulfoxide, THF = tetrahydrofuran, DIBAL = diisobutylaluminium hydride, PCC = pyridinium chlorochromate.

Syntheses of 10-Homonerol (2b) and 10-Homonerol Oxide (1b)

(Z)-2-Ethyl-6-methyl-1-iodo-1,5-heptadiene (**6**). 1-Bromo-4-methyl-3-pentene (**4**, 2.64 g, 16.2 mmol) in dry ether (30 ml) was slowly added to Mg metal (480 mg) and a small crystal of iodine. After 1.5 hr at reflux, the solution was carefully transferred into a solution of $\text{CuBr}/\text{Me}_2\text{S}$ (3 g, 0.9 equiv) in 10 ml of ether and 20 ml dimethylsulfide at -55°C . This temperature was kept for 2.5 hr. Then 1-butyne (**5**, 1.26 ml, 870 mg) was added via a cooled syringe during 1 min at -45°C . The mixture was warmed to -23°C for 2 hr and then recooled to -40°C . Iodine was added in small portions and the mixture was allowed to warm to -10°C for 0.5 hr. Ammonium chloride (20 ml, 10% aq.) was added and the organic layer was diluted with pentane, separated, filtered and washed ($\text{Na}_2\text{S}_2\text{O}_3$, NH_3 aq., NaCl 10% aq.). This procedure yielded 2.6 g (61%) of the desired product **6**; [^1H]NMR (200 MHz) δ 1.0 (t, 3H), 1.6 (s, 3H), 1.7 (s, 3H), 2.0–2.4 (m, 6H), 5.1 (t, 1H), and 5.9 (bs, 1H).

(Z)-3-Ethyl-7-methyl-2,6-octadienoic Acid (**7**). An ether solution of *n*-butyllithium (1.2 M, 1.14 mmol) was added under stirring at -70°C to the iodoalkene **6** (1 mmol) in ether (10 ml). The mixture was stirred at -60°C for ca. 30 min. The reaction was complete when the Gilman test was negative (Fieser and Fieser, 1967). Then excess carbon dioxide was bubbled through the mixture at -78°C . The reaction mixture was allowed to warm to -20°C . Water (5 ml) was added followed by an aqueous sodium bicarbonate solution until the mixture was alkaline. The aqueous layer was then acidified with hydrochloric acid (1 M) followed by extraction with ethyl ether (3×10 ml). The organic solution was dried (MgSO_4) and the solvent evaporated to give the acid **7**, 62 mg (34%); [^1H]NMR (200 MHz) δ 1.08 (t, 3H, $J = 7.4$), 1.62 (s 3H), 1.69 (s, 3H), 2.10–2.26 (m, 4H), 2.63 (t, 2H, $J = 7.4$), 5.15 (bt, 1H), 5.66 (bs, 1H), and 10.7 (broad, 1H).

(Z)-3-Ethyl-7-methyl-2,6-octadien-1-ol, 10-Homonerol (**2b**). A solution of the acid **7** (62 mg, 0.34 mmol) in anhydrous ethyl ether (5 ml) was added dropwise to lithium aluminium hydride (25.8 mg, 0.68 mmol) in anhydrous ethyl ether (5 ml) to create a gentle reflux. After 2 hr, sodium sulfate decahydrate-celite was added (Baekström, 1978). After another 1.5 hr, the solid was filtered off and washed with ethyl ether. After evaporation of the solvent and flash-chromatography, the combined ether filtrates gave the 10-homonerol (**2b**),

30 mg (45%); MS (*m/e*) 168 (M), 150 (M-18), 135 (M-18-15), 121, 107, 79, 69 and 41; [^1H]NMR (200 MHz) δ 1.03 (t, 3H, $J = 7.4$), 1.60 (s, 4H), 1.69 (s, 3H), 1.9-2.1 (m, 6H), 4.13 (d, 2H, $J = 7.0$), 5.11 (bt, 1H), and 5.42 (t, 1H).

Photooxidation of 10-Homonerol (2b). 10-Homonerol (**2b**, 30 mg, 0.18 mmol) was dissolved in a chloroform solution of Rose Bengal (3 ml). The solution was irradiated in a Rayonet reactor equipped with 16 RPR 350 nm lamps. Oxygen was bubbled through the solution. Tetrabutylammonium borohydride (46 mg, 0.18 mmol) was added in three portions (23, 12, and 11 mg, respectively) after 0, 60, and 80 min of irradiation (Baeckström et al., 1982). After 1.5 hr, the chloroform was removed by evaporation. Potassium iodide (60 mg, 0.36 mmol) in water (1 ml) and ethyl ether (6 ml) was then added to the residue. After stirring for 10 min, the resulting crystals were removed and washed by filtration. The combined red ethereal layer containing the alcohols **8** and **9** was separated from the aqueous phase and dried (MgSO_4).

10-Homonerol Oxide (1b). The obtained ethereal solution of the alcohols **8** and **9** was mixed with 2 drops of perchloric acid in ether (4 ml) and stirred for 30 min. After addition of a small amount of sodium carbonate, the resulting crystals were filtered off and washed with ethyl ether. Silica gel (2 g) was added to the ethyl ether solution and the solvent was evaporated in vacuo. The red powder with the adsorbed reaction products was added to the top of a column for flash-chromatography on basic alumina. 10-Homonerol oxide was obtained in a pure state. The GC-MS and [^1H]NMR spectra were identical with those of the natural compound (cf. Figures 2 and 3). [^1H]NMR (200 MHz) δ 1.01 (t, 3H, $J = 7.5$), 1.69 (d, 3H, $J = 1.2$), 1.73 (d, 3H, $J = 1.2$), 1.85-2.16 (m, 4H), 4.19 (m, 3H), 5.22 (bd, 1H), and 5.39 (bs, 1H).

Syntheses of Nostrenol (3) and Related Alcohols 10 and 11

6-Methyl-6-hexanolide (18). *m*-Chloroperbenzoic acid (11.9 g) in methylene chloride (100 ml) was added to a stirred solution of 2-methylcyclohexanone (5.6 g) in methylene chloride (20 ml). The resulting mixture was refluxed for 2 hr, cooled to 0°C, filtered and the filtrate was concentrated in vacuo. Bulb-to-bulb distillation (10 mm Hg/air bath at 100-120°C) gave an oil (4.70 g, 73%). NMR (60 MHz) δ 1.4 (d, 3H), 1.4-2.1 (m, 6H), 2.5-2.8 (m, 2H), and 4.1-4.8 (m, 1H).

Cyclic Lactols 12, 13, and 14. One of the lactones **18**, **19**, or **20** (0.1 mol) was stirred in dry THF (100 ml) at -50°C under argon. DIBAL (25 ml, 0.21 mol) in THF (100 ml) was added (30 min) and stirring at -50°C was continued for an additional 30 min. A powdered mixture of celite (1 vol), sodium sulfate decahydrate (70 g, 1 vol), and ethyl ether (300 ml, saturated with water) was added at -50°C. The mixture was allowed to warm slowly to room tempera-

ture, after which it was filtered and the solid was thoroughly washed with ethyl ether. The filtrate was dried (MgSO_4) and concentrated to give an oil (9.5–10.1 g, 93–99%), which was used in the next step without further purification.

Wittig Reaction of Cyclic Lactols 12, 13, and 14. Sodium hydride (300 mg, 80% in oil, 20 mmol) was washed free of oil with dry THF in portions under argon. The residual THF was evaporated under vacuum and the flask filled with argon. DMSO (40 ml, freshly distilled from CaH_2) was added, and the mixture was stirred and heated slowly to 60°C until all the hydride had reacted. After cooling to 20°C an alkyltriphenylphosphonium bromide (20 mmol) was added to the stirred solution. The ylide (**15**, **16**, or **17**) was formed rapidly, and the resulting intensely red solution was cooled to 15°C and a solution of the lactol (**12**, **13**, or **14**, 20 mmol) in DMSO (10 ml) was added dropwise. After stirring at 15°C for 1.5 hr, the reaction mixture was poured into water and extracted with pentane. The pentane solution was concentrated to 10 ml and filtered. The solid was washed with a few milliliters of pentane, and the solution was concentrated to give an oil, which was subjected to flash-chromatography to give the (*Z*)-alkenol (**10**, **11**, or **21**) after bulb-to-bulb distillation (air bath at $130^\circ\text{C}/7$ mm Hg, yield 14–16 mmol, 70–80%). The products contained less than 8% of the *E*-isomers (GC). The NMR spectra of **10** and **11** are shown in Figure 5.

(*Z*)-5-Decen-1-ol (**21**). Bp $58\text{--}60^\circ\text{C}/1$ mm Hg. GC showed the ratio (*Z*/*E*) to be 93/7; n_{25}^D 1.4504; IR (film) ν_{max} 3320 cm^{-1} (very broad, H-bonded OH); NMR (60 MHz) δ 0.9 (t, 3H), 1.1–1.8 (m, 8H), 1.8–2.3 (m, 4H), 2.80 (s, 1H disappears on addition of D_2O), 3.60 (t, 2H), and 5.37 (m, 2H).

(*Z*)-5-Decenal (**22**). (*Z*)-5-Decen-1-ol (**21**, 5 g, 32 mmol) was stirred in methylene chloride (20 ml). PCC (16 mg, 50 mmol) in methylene chloride (100 ml) was added. The mixture was stirred at room temperature for 2 hr, poured into ethyl ether (1.5 l), and the organic phase was washed with aqueous HCl (2 M) followed by saturated sodium bicarbonate solution and water. The organic phase was dried (MgSO_4) and concentrated to give an oil, which, after flash-chromatography and distillation (bp $80\text{--}85^\circ\text{C}/7$ mm Hg), furnished the pure aldehyde (4.3 g, yield 87%, 99% pure, *Z*/*E* = 93/7 by GC); n_{25}^D 1.4491; IR (film) ν_{CO} 1725 cm^{-1} (strong); NMR (60 MHz) δ 0.93 (t, 3H), 1.1–2.2 (m, 10H), 2.43 (dt, 2H, $J = 6.8$ and 1.6), 5.40 (m, 2H), and 9.82 (t, 1H, $J = 1.6$).

Nostrenol, rac-(Z)-6-Undecen-2-ol (3). Methyllithium (1.6 M in ethyl ether, 50 ml) was stirred in dry ethyl ether (100 ml) at -80°C under argon. (*Z*)-5-Decenal (**22**, 4.1 g) in ethyl ether (25 ml) was added dropwise. After stirring for 2 hr, the temperature was raised to -50°C and 2 M aqueous HCl was added. After warming to room temperature the reaction mixture was poured into water. The organic phase was washed with water, dried (MgSO_4), and the solvent evaporated to give an oil, which was flash-chromatographed and dis-

tilled (3.2 g, yield 72%, bp 108–109°C, 12 mm Hg, 99% pure, *Z/E* = 95/5 by GC). This compound had identical IR, [¹H]NMR, and MS spectra and GC behavior (achiral columns) as those of natural nostrenol.

Syntheses of (S)- and (R)-Nostrenol (26 and 28, respectively)

(Z)-1-Chloro-3-octene (25). (Z)-3-Octen-1-ol (**23**, 2.62 g, 20.5 mmol; Zhong et al., 1982), triphenylphosphine (6 g, 23 mmol) and carbon tetrachloride (4 ml) were mixed and heated to 120°C (cf. Hooz and Gilani, 1968). A vigorous reaction occurred. Heating was continued for 15 min. Ethanol (0.5 ml) was added and heating was continued for an additional 15 min. After cooling, pentane (25 ml) was added. Precipitated triphenylphosphine oxide was filtered off and the filtrate was carefully concentrated to give an oil, which, on addition of pentane (10 ml), precipitated further oxide that was removed, and the pentane was evaporated to give an oil. This was chromatographed on silica gel. Pentane eluted the desired product, which was distilled to give 2.92 g (88%). Bp 60–61°C/5 mm Hg; n_{25}^D 1.4486; [¹H]NMR (60 MHz) δ 0.93 (t, 3H), 1.2–1.7 (m, 4H), 1.9–2.3 (m, 2H), 2.54 (quint., 2H, *J* = 7.5), and 5.48 (t, 2H, *J* = 7.5).

(S)-Nostrenol, (S)-(Z)-6-Undecen-2-ol (26). (Z)-1-Chloro-3-octene (**25**, 2.94 g, 20 mmol) in dry THF (15 ml) was added to magnesium turnings (0.72 g, 30 mmol), which had been activated by a few drops of 1,2-dibromoethane under argon. The resulting mixture was heated to reflux for 1 hr. After cooling, the solution of the Grignard reagent was decanted from the excess magnesium and added via a syringe to a solution of CuBr/Me₂S (1 mmol) in THF (20 ml) at –30°C under argon (cf. Huynh et al., 1979). (S)-Epoxypropane (**24**, 1.2 g, 21 mmol) was added. After stirring at –30°C for 1 hr, the temperature was raised to 20°C. The reaction mixture was poured into an aqueous solution of ammonium chloride. The resulting mixture was diluted with pentane and the aqueous layer extracted twice with pentane. The combined organic phases were washed with water, dried (MgSO₄), and the solvent was carefully evaporated to give 2.72 g (80%) after flash-chromatography and distillation, bp 108–109°C/12 mm Hg; [α]_D = +6.02° (neat); *Z/E* ratio > 98.5/1.5 by GC (Carbowax 20 M, 135°C). The [¹H]NMR spectrum (200 MHz) was identical with that of natural nostrenol.

(R)-Nostrenyl benzoate, (R)-(Z)-6-Undecen-2-yl benzoate (27). (S)-Nostrenol (**26**, 1.70 g, 10 mmol), triphenylphosphine (6.55 g, 25 mmol), and benzoic acid (3.05 g, 25 mmol) was stirred in dry THF (100 ml) under argon at 0°C. Diethyl azodicarboxylate (4.52 g, 26 mmol) in THF (10 ml) was added during 5 min. After stirring overnight at room temperature, methanol (0.6 ml) was added to destroy excess reagents. After concentration to 25 ml, the mixture was poured into pentane. The precipitate was removed by filtration and the

solvent evaporated. The remaining oil was flash-chromatographed. Ethyl acetate (2–3%) in light petroleum eluted 2.15 g (78%), pure by GC; $[\alpha]_D = -26.4^\circ$ (neat); ^1H NMR (60 MHz) δ 0.9 (t, 3H), 1.0–2.2 (m, 12 H), 1.37 (d, 3H), 5.2 (sextet, 1H), 5.3 (m, 2H), and 7.2–8.2 (m, 5H).

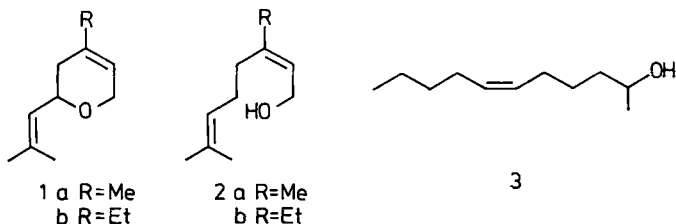
(*R*)-*Nostrenol*, (*R*)-(*Z*)-6-Undecen-2-ol (**28**). (*R*)-*Nostrenyl benzoate* (**27**, 1.67 g, 0.61 mmol) was dissolved in dry ether (25 ml) under argon. A suspension of lithium aluminium hydride in ether (1 M, 20 ml) was added. The mixture was stirred overnight, when aqueous HCl (2 M) was carefully added to pH 1. After extraction twice with pentane, the organic phase was washed with water, dried (MgSO_4), and concentrated to give an oil, which was flash-chromatographed. Ethyl acetate (3–4%) in light petroleum eluted a colorless oil, which was distilled (Kugelrohr 10 mm Hg/air bath 130°C), 0.9 g (87%); $[\alpha] = -6.08^\circ$ (neat).

rac-(*E*)-6-Undecen-2-ol. This compound was prepared via the reaction sequence described for (*S*)-(*Z*)-nostrenol (**26**) except that the starting materials were *rac*-(*E*)-3-octen-2-ol (Zhong et al., 1982) and racemic epoxypropane; colorless oil, ^1H NMR (60 MHz) δ 0.85 (t, 3H), 1.10 (d, 3H) 1.1–2.2 (m, 9H), 3.8 (m, 1H), and 5.3 (m, 2H); IR (film) 970 cm^{-1} [(*E*)-CH = CH-].

RESULTS

Identification

Typical capillary gas chromatograms of the volatile secretions from *E. nostras* and *G. bore* are given in Figure 1. The two major components in each species were nerol oxide (**1a**) and (*Z*)-6-undecen-2-ol (nostrenol, **3**) in *E. nostras*, and 10-homonerol oxide (**1b**) and nostrenol (**3**) in *G. bore* (Scheme 1). In both species these compounds are found in the proportions 3:2 (in the order mentioned above). The small peaks present in the gas chromatograms emanate from the solvent used for extraction (hydrocarbon isomer and phthalates). Similarly, the major components of *M. formicarius* were found to be nerol (**2a**) and 10-homonerol (**2b**).



SCHEME 1.

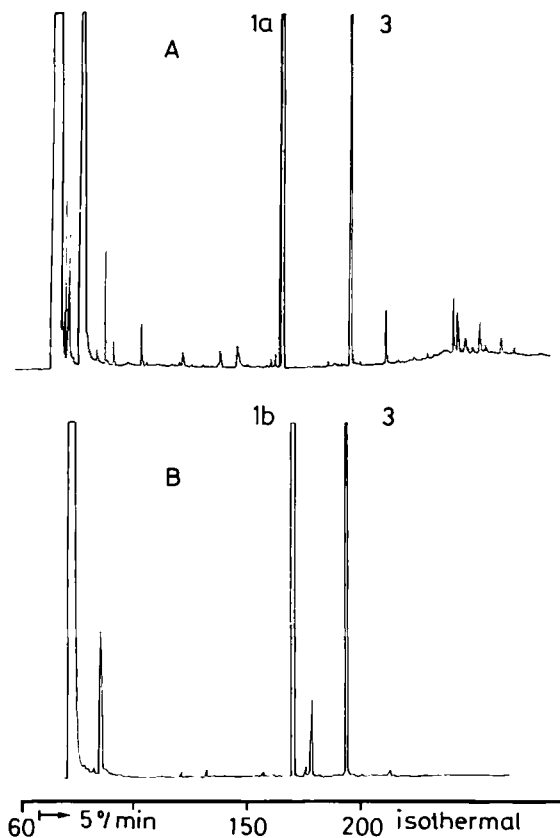


FIG. 1. Capillary gas chromatographic separation of the volatile compounds from the thoracic glands of *E. nostras* (A) and *G. bore* (B). Column: Superox FA 27 m \times 0.25 mm. Temperature program: isothermal at 60°C for 2 min and then programmed to 200°C at a rate of 5°C/min.

Structure Determinations

10-Homonerol Oxide (1b). This compound, present in the secretion from males of *G. bore*, has a mass spectrum ($m/e = 166$) very similar to that of nerol oxide (1a). Löfqvist and Bergström (1980) suggested that it was a homonerol oxide. This was confirmed by a methyl triplet present in the [^1H]NMR spectrum, which indicates that the structure can be 10-homonerol oxide (1b) with an ethyl substituent instead of the methyl group in the 3-position (Figures 2 and 3). The other possible isomers, 8- and 9-homonerol oxide (Rohjahn and Bruhn,

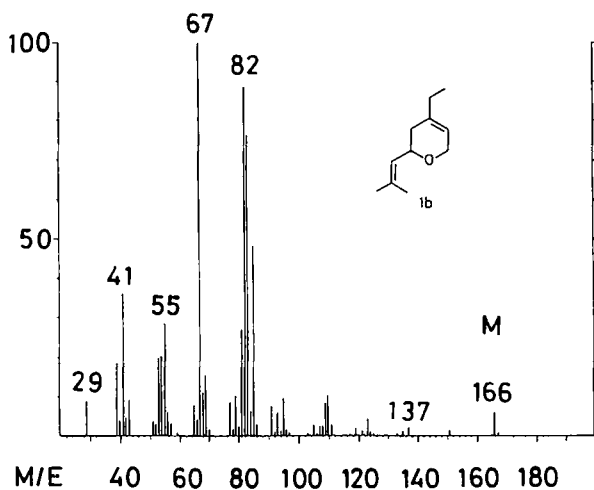


FIG. 2. Mass spectrum of natural 10-homonerol oxide (**1b**) from *G. bore*.

1978), were not identical with the natural product as shown by comparisons (GC-MS; NMR) with an authentic sample of a mixture of the two isomers.

In order to confirm the structure of 10-homonerol oxide (**1b**) a synthesis was designed according to Scheme 2. The alkyl cuprate of **4**, prepared from the

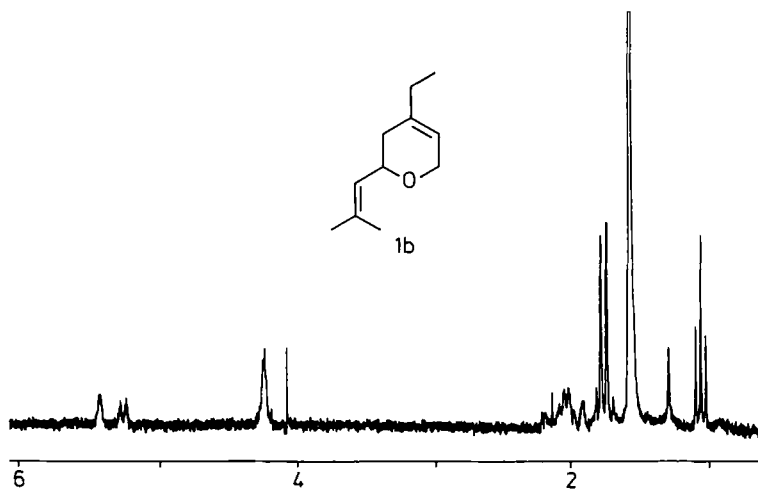
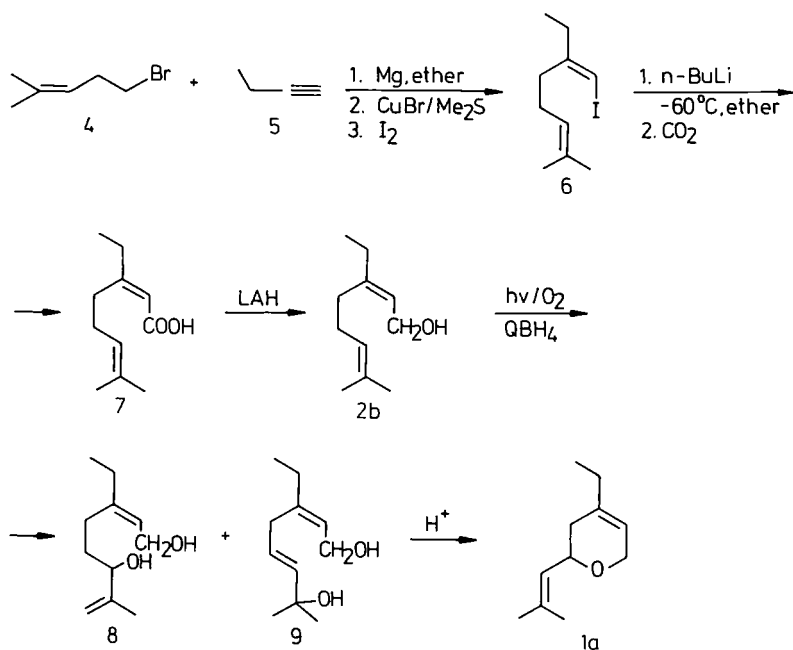


FIG. 3. ^1H NMR of natural 10-homonerol oxide (**1b**); approx. 50 μg (in CDCl_3) derived from an extract of 45 males of *G. bore*. The large peak at δ 1.54 belongs to water.

corresponding magnesium reagent, was added to 1-butyne (**5**) followed by treatment with iodine to form the vinyl iodide **6**. Lithiation of **6** followed by alkylation with carbon dioxide yielded the acid **7** (Cahiez et al., 1976). Reduction with lithium aluminium hydride (LAH) afforded the 10-homonerol (**2b**). Photooxidation with singlet oxygen of **2b** and simultaneous reduction with tetramethylammonium borohydride (QBH₄; Bäckström et al., 1982) gave the two isomeric alcohols **8** and **9**. Acidic cyclization of the alcohol mixture (Ohloff et al., 1980) gave the 10-homonerol oxide (**1b**), which was separated from the unreacted alcohol by column chromatography. The GC-MS data and the [¹H]NMR spectrum synthetic **1b** were identical with those of the isolated natural product of *G. bore*.



SCHEME 2.

10-Homonerol (2b). From *M. formicarius* we have earlier identified nerol (**2a**) in the male volatile secretion. A second compound gave $m/e = 168$ (Löfqvist and Bergström, 1980). It has now been identified as 10-homonerol (**2b**) by comparison of GC-MS data (magnetic and quadropole instrument) of the natural substance and the synthetic compound produced as an intermediate in the synthesis of 10-homonerol oxide.

(*Z*)-6-Undecen-2-ol (*Nostrenol*, **3**). This compound was found to be present in the secretions of *E. nostras* and *G. bore*, as shown by identical mass spectra (Figure 4) and GC retention times (Figure 1). It was identified by [¹H]NMR as an undecen-2-ol, which was named *nostrenol* (**3**).

The spectral data gathered for *nostrenol* (**3**) were inconclusive regarding the double bond position. However, preliminary inspection of the [¹H]NMR spectrum suggests the most probable isomers to be **3**, **10**, and **11**. The synthetic procedures of those isomers are outlined in Scheme 3. The key step is the Wittig reaction of the lactols **12**, **13**, and **14** with the appropriate alkylidene phosphoranes **15**, **16**, and **17**, respectively. Wittig reactions in dimethylsulfoxide are known to give a high ratio of *Z* to *E* configuration (Goto et al., 1975; Hall et al., 1975), and this was also found in our case (*Z/E* > 10:1). The lactols **12**, **13**, and **14** were prepared in excellent yields by reduction of the corresponding lactones **18**, **19**, and **20** with diisobutylaluminum hydride in tetrahydrofuran. The lactone **18** was obtained from 2-methylcyclohexanone on oxidation with *m*-chloroperbenzoic acid.

Racemic compound **3** was prepared from (*Z*)-5-decenol (**21**) via oxidation to (*Z*)-5-decenal (**22**) by pyridinium chlorochromate. The aldehyde was then reacted with excess methylolithium in ethyl ether at low temperature which furnished (*Z*)-6-undecen-2-ol (**3**).

The [¹H]NMR spectra of the (*Z*)-undecen-2-ols **3**, **10**, and **11** as well as that of the natural product isolated from *E. nostras* are shown in Figure 5. From

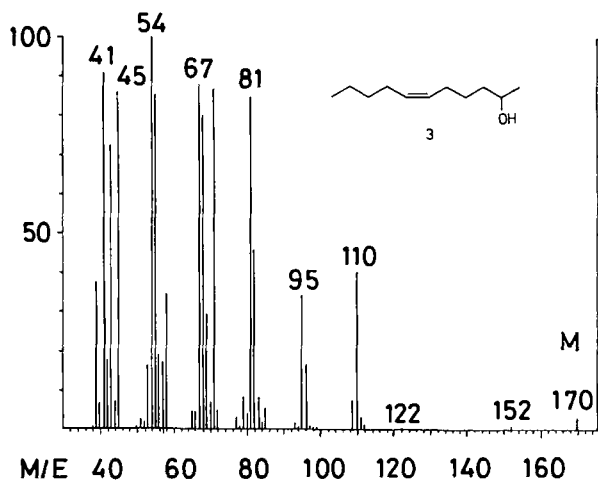
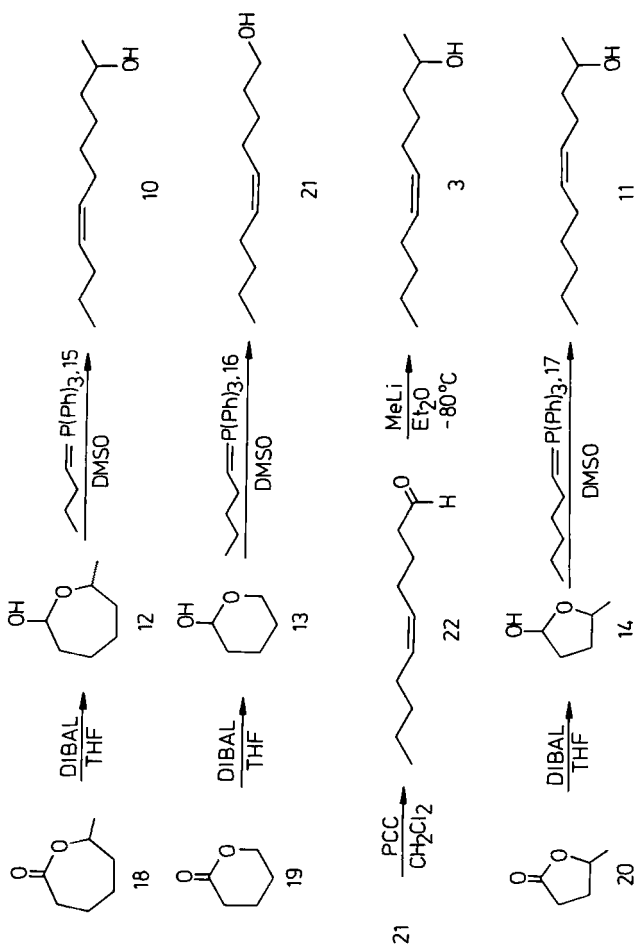


FIG. 4. Mass spectrum of natural *nostrenol* (**3**) from *E. nostras*, also present in *G. bore*.



SCHEME 3.

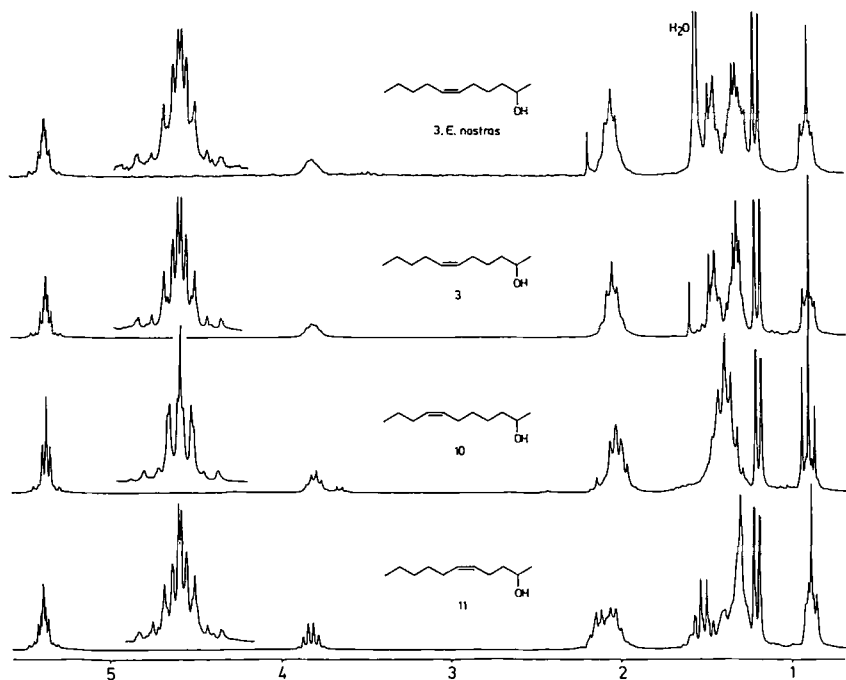


FIG. 5. ^1H NMR spectra of extract from *E. nostras* (top), (*Z*)-6-undecen-2-ol (**3**), (*Z*)-7-undecen-2-ol (**10**), and (*Z*)-5-undecen-2-ol (**11**) (bottom). For comparison the signals from the vinylic protons are expanded.

the comparison of these spectra it is evident that (*Z*)-6-undecen-2-ol (**3**) is identical with the natural nostrenol.

Absolute Configurations

Nerol oxide (1a). It was of interest to investigate whether the identified compounds from the ant-lions were optically active and if there was any difference in chirality between the three species. Samples of nerol oxide with known absolute configurations (Ohloff et al., 1980) were investigated. $\text{Eu}(\text{HFC})_3$ was added to (*S*)-nerol oxide ($L/S = 0.5$), and the ^1H NMR showed that the low-field doublet at δ 5.6–5.8 belonged to H-7 of (*S*)-nerol oxide. When a solution of racemic nerol oxide and $\text{Eu}(\text{HFC})_3$ ($L/S = 0.5$) was added to the sample of (*S*)-nerol oxide and $\text{Eu}(\text{HFC})_3$, the ratio between the two doublets changed according to the ratio between the *S*-enantiomer and the racemate. Thus (*R*)-

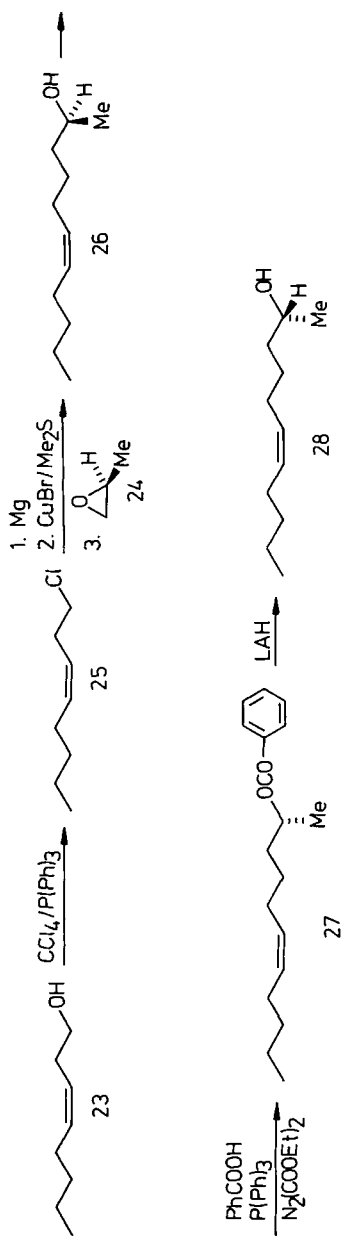
nerol oxide gives rise to the upfield doublet in this region. Addition of 0.45 equivalents of $\text{Eu}(\text{HFC})_3$ to naturally occurring nerol oxide (200 μg) from *E. nostras* resulted in two signals from H-7 in a 1 : 1 ratio, showing that this natural nerol oxide is racemic.

The nerol oxide from *E. nostras* was also analyzed using fused silica capillary GC columns coated with either Ni(II)- or Mn(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate] (Schurig and Weber, 1981, 1984). The results confirmed that the nerol oxide is racemic. (*S*)-Nerol oxide was used as reference. The elution order of the *S*-enantiomer compared to that of the *R*-enantiomer was reversed on the two columns (see Methods and Materials).

10-Homonerol Oxide (1b). The homonerol oxide isolated from *G. bore* was similarly tested and found to be racemic. However, only the Mn(II)-derived chiral phase separated the enantiomers of **1b**. The close relationship between **1a** and **1b** suggests also that (*S*)-10-homonerol oxide has the shorter retention time (see Methods and Materials).

Nostrenol (3). In order to establish the chirality of natural nostrenol an asymmetric synthesis was outlined as shown in Scheme 4. (*Z*)-3-Octen-1-ol (**23**) (Zhong et al., 1982) and commercially available (*S*)-epoxypropane (**24**) were used as starting materials. The alcohol **23** was transformed into the chloride **25** on treatment with triphenylphosphine and tetrachloromethane (Hooz and Gilani, 1968). The chloride was converted to the alkyl cuprate via the corresponding Grignard reagent followed by reaction with (*S*)-epoxypropane (**24**) to furnish (*S*)-nostrenol (**26**). The *R*-enantiomer was prepared from (*S*)-nostrenol (**26**) via a Mitsunobu reaction (Mitsunobu, 1981). Thus, (*S*)-nostrenol (**26**) was reacted with triphenylphosphine, diethyl azodicarboxylate, and benzoic acid, which gave (*R*)-nostrenyl benzoate (**27**). Reduction with lithium aluminium hydride furnished (*R*)-nostrenol (**28**). Gas chromatography of the isopropyl carbamates of the two alcohols on a tandem GC column [SE-54/XE-60-(*S*)-valine-(*S*)-2-phenylethylamide] showed that the synthetic enantiomers were almost optically pure ($\approx 93\%$ ee) and contained less than 2% of the *E*-isomer. The latter was prepared as the racemate as described above but starting from (*E*)-3-octen-1-ol (Zhong et al., 1982) and racemic epoxypropane. Retention times were 118.9 min [(*Z*)-(*R*)-6-undecen-2-ol], 119.2 min [(*Z*)-(*S*)], 119.4 min [(*E*)-(*R*)], and 119.7 min [(*E*)-(*S*)] on the tandem GC column.

Nostrenol from *E. nostras* was analyzed by capillary GC as the isopropyl carbamate derivative. The naturally occurring sample was found to be enantiomerically pure ($>99.9\%$) and of *R*-configuration. The nostrenol isolated from *G. bore* was also found to be of *R*-configuration. The retention times were 66.4 min and 66.9 min for the (*R*)- and (*S*)-nostrenols, respectively, on the column coated with XE-60-(*S*)-valine-(*S*)-2-phenylethylamide.



SCHEME 4.

DISCUSSION

The ant-lion species are old from an evolutionary point of view. Therefore, it is of interest to investigate the pheromone systems of these insects in order to gain knowledge of possible isolating mechanisms between closely related species. The male ant-lion species have large thoracic glands, producing characteristic volatile chemicals which exhibit close structural relationships between the different species. The adults live only for a few days during which they must mate. These observations suggest that the substances may form part of a communication system associated with mating and contributing towards isolating mechanisms between the sympatric species.

Löfqvist and Bergström (1980) reported earlier that the thoracic glands of each of the three ant-lion species contained two major volatile constituents. One of the compounds in *E. nostras* was found to be nerol oxide (**1a**), while nerol (**2a**) was identified from *M. formicarius*. In the present investigation we have used [¹H]NMR and/or MS to identify the three remaining components as 10-homonerol oxide (**1b**) in *G. bore*, 10-homonerol (**2b**) in *M. formicarius*, and (*Z*)-6-undecen-2-ol (nostrenol, **3**) in *E. nostras* and *G. bore*. Four of these compounds are biosynthetically related and of isoprenoid origin, whereas the fifth compound, nostrenol (**3**), is of another biogenetic origin. It seems that the three sympatric ant-lion species make use of a combination of a minimum of two substances for reproductive isolation.

Nerol (**2a**) and nerol oxide (**1a**) are well-known plant constituents while their homologs 10-homonerol (**2b**) and 10-homonerol oxide (**1b**) have not been identified previously. Nostrenol (**3**) has been reported as a minor constituent of cognac (ter Heide et al., 1978), but its synthesis and spectral data have not, to our knowledge, been reported in the literature.

Syntheses were designed for 10-homonerol, 10-homonerol oxide, and nostrenol in order to verify the structural assignments and for EAG measurements and bioassays. The biological role of the compounds is currently under investigation.

Three of the isolated compounds possess an asymmetric center, and we have found that nostrenol from the two ant-lion species *E. nostras* and *G. bore* is enantiomerically pure and of *R*-configuration. The nerol oxide of *E. nostras* as well as the closely related 10-homonerol oxide of *G. bore* are racemic. Nerol oxide has not yet been found to occur in nature in a chiral form (Ohloff et al., 1980).

A GC separation of a mixture of *Z*- and *E*-isomers of racemic 6-undecen-2-ols into their chiral entities was successfully performed by connecting one column for the separation of geometrical isomers to a chiral column for subsequent separation into the two pairs of enantiomers. The corresponding isopropyl carbamates were employed for this separation, and use of a nitrogen-

sensitive flame photometric detector gave a sensitivity 10–20 times greater than that with a flame ionization detector.

The amount of the compounds isolated from *E. nostras* was relatively large compared with amounts of pheromone components typically found in insects. Thus, it was possible to obtain a large enough amount of material to determine the chirality of nerol oxide (200 μg) by [^1H]NMR after addition of a chiral shift reagent. The chirality of nerol oxide was also determined in the nanogram range by GC on a column coated with Ni(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate]. This chiral phase could not, however, separate the enantiomers of 10-homonerol oxide. On the other hand the corresponding Mn(II)-derived GC phase separated the enantiomers of both nerol oxide and 10-homonerol oxide. Furthermore, the order of elution of the two enantiomers of nerol oxide was reversed on these two chiral GC phases.

The NMR technique for structural and configurational studies has rarely been used on insect pheromones, which are generally produced only in nanogram quantities. Silverstein and coworkers (Plummer et al., 1976; Stewart et al., 1977) have reported the enantiomeric composition of a few pheromones (alcohols and ketals) determined by the addition of shift reagents. The quantity (150–200 μg) of compound used in these and the present investigation is at present the minimum amount for determination of chirality by addition of a chiral shift reagent.

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HPLC IDENTIFICATION OF ALLELOPATHIC COMPOUNDS FROM *Lantana camara*¹

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Abstract—Aqueous extracts of *Lantana camara* L. leaves inhibited ryegrass (*Lolium multiflorum* Lam.) germination and seedling growth. Phytotoxic compounds were fractionated from crude aqueous extracts and fractions were evaluated for their phytotoxicity. Inhibition was most pronounced with the alkaline and acid hydrolysates. Plant inhibition by the crude extract reflected a complex interaction of numerous individual components of diverse chemical compositions and potencies. Presumptive identification of the individual components was accomplished with high-performance liquid chromatography (HPLC). Thirteen phenolic compounds were identified, and most of these compounds were phytotoxic to ryegrass seedlings. Radicle elongation was more sensitive to the toxins than shoot elongation.

Key Words—Allelopathy, *Lantana camara*, ryegrass, phenolic compounds.

INTRODUCTION

Lantana camara L., a widespread weed in many citrus groves in Florida, is also one of the world's worst weeds (Holm et al., 1977). Its leaves and seeds are toxic to many animals (McSweeney and Pass, 1982; Sharma et al., 1981) and humans (Mortan, 1971). Habeck (1976) suggested that lantana competes with citrus for nutrients, but there is no experimental evidence to substantiate this claim. Pope et al. (1983), Rice (1984), Achhireddy and Singh (1984), Achhireddy et al. (1985), and Mersie and Singh (1987) previously reported the

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effect of lantana extracts on various weed and crop species. The nature and characteristics of the inhibitory compounds were not determined. This study deals with the isolation and identification of allelochemicals (particularly phenolics) in fractions of lantana extracts and the gross determination of their biological activity on ryegrass (*Lolium multiflorum*).

METHODS AND MATERIALS

Materials. Leaves of lantana were collected in the vicinity of Lake Alfred, Florida. Phenolic standards used in this study were obtained from Sigma Chemical Company (St. Louis, Missouri). Solvents were from Burdick and Jackson Laboratories, (Muskegon, Michigan).

Extraction and Isolation of Phytotoxins. Methanol, methylene chloride, and water extracts (1000 ml each) of 100 g fresh lantana leaves were preliminarily tested on ryegrass seed growth. Concentration equivalents of 5, 10, and 25 mg of fresh leaf tissue/Petri dish were used in the ryegrass bioassay.

Since water is the extraction solvent in nature, the procedures were designed to allow a natural release of toxins. A crude aqueous extract was prepared by soaking 100 g of fresh leaf material in 1000 ml of distilled water for 48 hr. The liquid was decanted, the leaves were reextracted twice by soaking the leaf residue in 100 ml distilled water for 24 hr, and the extracts combined. The aqueous extract was filtered through cheesecloth and the solids discarded. The filtrate was centrifuged for 15 min at 3000 rpm and the supernatant decanted. The residue was washed twice with 50 ml of water, centrifuged, and all supernatants were combined. The final filtrate was concentrated to 1000 ml in vacuo below 40°C.

The aqueous extract (fraction 1) obtained as above was first extracted 3× with 100 ml of hexane to remove lipids (fraction 2). The resulting aqueous solution (fraction 3) was extracted with diethyl ether (fraction 4). The remaining aqueous fraction (fraction 5) was subjected separately to acid and alkaline hydrolyses. Acid hydrolysis was carried out in the dark in a water bath at 40°C for 2 hr in 2 N HCl. The hydrolysate was extracted with diethyl ether (fraction 8). Alkaline hydrolysis was also carried out in the dark with 2 N NaOH for 2 hr under nitrogen at room temperature and the hydrolysate extracted with ethyl ether (fraction 6). All extractions with ethyl ether (100 ml) were repeated three times. The severe acid and alkaline hydrolyses were employed to release the potential allelochemical moieties from water soluble conjugates. Each of these fractions, along with the remaining aqueous fractions from alkaline (fraction 7) and acid (fraction 9) hydrolyses, was used for bioassay as described below (Figure 1).

Tests for Phytotoxicity. Phytotoxicity of the various lantana fractions was

determined through germination assays using ryegrass seeds. Two Whatman No. 1 filter papers in 9-cm glass Petri dishes were initially wetted with 5 ml of treated solution at concentrations equivalent to 5, 10, and 25 mg of leaf fresh weight/Petri dish. After the solvent evaporated, 10 ryegrass seeds were placed in each dish and 5 ml of glass-distilled water were added. The experiment was conducted in the laboratory at 25°C with a 12-hr photoperiod (150 $\mu\text{E}/\text{m}^2/\text{sec}$). Control treatments received 5 ml of glass-distilled water. After seven days, growth was determined by measuring the lengths of roots and shoots. Seeds were considered germinated if the radicle had emerged from the seed coat. The experiment was repeated twice in triplicate sets.

Analytical Method. Gradient separation (Table 1) and identification of the different fractions was accomplished on a high-performance liquid chromatograph (Waters Associates, Milford, Massachusetts), model 6000 A pump, equipped with a U6K injector. Elution was monitored at 280 nm using a variable wavelength Perkin Elmer LC 75 spectrophotometric UV detector. All injections were 10 μl ; samples were filtered through a 0.45- μm filter prior to injection into the chromatograph. Separation by HPLC was done by using a reverse-phase C 18- μ Bondapak column (7.8 mm \times 30 cm). A Waters 730 Data Module and a 721 System Controller were used for retention times and peak areas. Presumptive identification of allelopathic compounds was done by comparison of retention times of unknown peaks with retention times of authentic standards.

Biological Activity of Identified Compounds. Phytotoxicity of each of the identified compounds was further verified with a 100 ppm solution using the bioassay procedure described above.

Data Analyses. The experiment was a randomized design with three replications for each treatment. Data from each experiment were expressed as per-

TABLE 1. GRADIENT HPLC CONDITIONS

Time (min)	Flow (ml/min)	Acetonitrile (pump %)	1% acetic acid in water (pump %)	Waters™ curve
0	1	60	40	
3	1	65	35	convex
5	1	70	30	convex
10	1	75	25	linear
15	1	80	20	linear
20	1	85	15	linear
25	1	90	10	linear
30	1	60	40	linear

Waters™. Waters HPLC curve convex = 5, Linear = 6. This is a programmed curve by Waters Associates (Milford, Massachusetts).

cent of the untreated control and then were combined (six replications per compound/fraction per concentration). The statistical model was a one-way analysis of variance, which allowed the means for fraction/standards and concentration to be pooled.

RESULTS AND DISCUSSION

Preliminary results showed inhibitory activity of methanol, methylene chloride, and water extracts on the growth of ryegrass roots and shoots by all three concentrations (5, 10, and 25 mg fresh weight/petri dish) of leaf extracts (Table 2). Water extracts were slightly less inhibitory than the extracts by the organic solvents. The inhibitory effect was more pronounced on root growth.

The aqueous leaf extract exhibited phytotoxic effects on ryegrass growth and seed germination (Table 3). A stepwise separation of the crude extract (Figure 1) and the various fractions resulted in a diminishing activity with some fractions. Fraction 3 (crude extract minus lipids) was as effective as the crude extract. The phenolic acids (fractions 6 and 8) and aglycones (fraction 4) all reduced germination of the test species, but the effect was slightly greater with fraction 6 (alkaline hydrolysate). This may be because more phenolic acids were liberated with alkaline hydrolysis of the water extract than with acid hydrolysis. The 2 N HCl will cleave most of the glycosidic linkages and 2 N NaOH at room temperature will cleave esters. The aqueous fractions from acid (fraction 9) and alkaline (fraction 7) hydrolyses inhibited ryegrass root growth slightly. The different leaf fractions varied in their ability to affect germination and growth of ryegrass. The basic (fraction 6) and acidic (fraction 8) organic fractions were the most active (Table 3; Figure 1).

TABLE 2. INHIBITION OF ROOT AND SHOOT LENGTHS OF RYEGRASS BY METHANOL, METHYLENE CHLORIDE, AND AQUEOUS EXTRACTS OF LANTANA LEAVES

Concentration (mg fr. wt./ plate)	Length (% of control) ^a					
	Root			Shoot		
	MeOH	CH ₂ CL ₂	H ₂ O	MeOH	CH ₂ CL ₂	H ₂ O
0	100.0	100.0	100.0	100.0	100.0	100.0
5	63.3	67.9	71.6	88.9	91.6	92.1
10	54.1	56.2	59.3	60.9	65.7	72.2
25	7.9	8.6	10.8	21.2	30.6	31.0
LSD (0.05)	8.8	7.9	7.4	6.5	6.8	8.1

^aThe average lengths of roots and shoots for control were 2.4 and 2.7 mm, respectively.

TABLE 3. EFFECT OF FRACTIONS OBTAINED FROM AQUEOUS EXTRACTS OF LANTANA LEAVES ON RYEGRASS

Fraction ^a	Concentration (mg fr. wt./plate)	% of control		
		Germination	Root length ^b	Shoot length ^b
1	5	100.0	69.5	93.1
	10	73.3	54.0	74.0
	25	58.0	11.3	29.5
2	5	100.0	100.4	98.2
	10	100.0	90.5	90.2
	25	96.1	81.8	91.6
3	5	100.0	60.9	93.8
	10	88.7	53.5	72.8
	25	60.0	15.6	30.3
4	5	94.0	73.5	93.0
	10	95.7	64.2	82.0
	25	96.6	29.6	71.2
5	5	97.0	81.9	94.7
	10	94.6	61.4	82.2
	25	96.6	20.3	50.3
6	5	100.0	82.2	95.9
	10	92.3	60.7	77.1
	25	80.3	21.6	43.6
7	5	100.0	103.5	95.9
	10	99.0	86.2	90.9
	25	100.0	61.9	88.7
8	5	96.6	88.4	95.5
	10	93.3	72.7	87.0
	25	85.3	29.2	68.6
9	5	100.0	93.9	96.8
	10	100.0	83.2	89.9
	25	97.7	70.7	87.7
LSD (0.05) ^c		11.06	1.27	1.07

^aSee Figure 1 for fractions 1-9.

^bThe average lengths of roots and shoots for control were 6.4 and 5.2 mm, respectively.

^cDifferences between fractions.

All fractions except fraction 2 (lipids) were toxic, to some extent, to ryegrass root and shoot growth. Toxicity depended upon concentration. Most extracts did not inhibit germination. Root growth was inhibited (69.5% of control) even at a low concentration of 5 mg fresh weight/Petri dish of the crude aqueous extract. Shoot growth was generally less affected. Previous studies (Achhireddy et al., 1985) indicated a greater sensitivity of root growth than shoot growth to inhibitors.

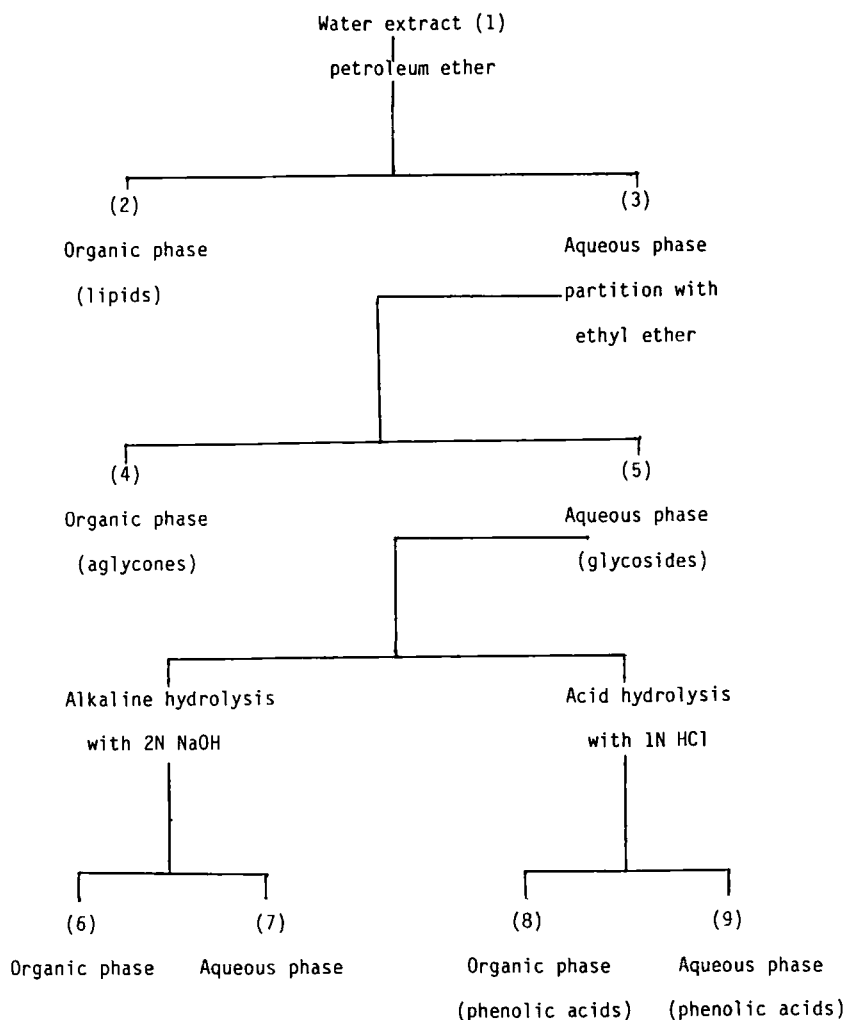


FIG. 1. Flow diagram for the separation of inhibitory compounds from the aqueous extract of *Lantana camara*.

The inhibitory basis of fractions 1 and 3 is probably the result of the combined expression of numerous components of diverse potencies, a phenomenon reported by several workers (Wilson and Rice, 1968; Muller and Chou, 1972; Lodhi, 1975). Phytotoxic effects on seed germination and seedling growth of ryegrass ranged from 58 to 100% inhibition for seed germination; 10–90% for root growth, and 10–50% for shoot growth (Table 3). The degree of inhibition

TABLE 4. INFLUENCE OF HPLC IDENTIFIED PHENOLIC COMPOUNDS (100 PPM) ON RYEGRASS GERMINATION AND SEEDLING GROWTH

Phenolic compounds Common name	Systematic name	Fraction ^e	HPLC retention time (min)	Mol wt	Conc. (mM)	Length (% control) ^b	
						Root	Shoot
Umbelliferone	<i>p</i> -hydroxycoumarin	organic	3.2	162.1	0.62	22.0	44.3
Methyl coumarin	6-methyl coumarin	organic	3.3	162.1	0.62	22.5	49.0
Genistic acid	2,5-dihydroxybenzoic acid	acidic	4.0	154.1	0.65	60.2	75.4
β -Resorcylic acid	2,4-dihydroxybenzoic acid	acidic and alkaline	4.9	154.1	0.65	42.4	64.2
α -Resorcylic acid	3,5-dihydroxybenzoic acid	acidic and alkaline	5.2	154.1	0.65	64.3	81.2
<i>p</i> -Hydroxybenzoic acid	4-hydroxybenzoic acid	acidic and alkaline	9.8	138.1	0.72	40.3	59.2
Salicylic acid	2-hydroxybenzoic acid	acidic and alkaline	11.4	138.1	0.72	22.4	48.1
Vanillic acid	4-hydroxy, 3-methoxybenzoic acid	acidic and alkaline	11.0	168.1	0.60	58.4	72.1
Caffeic acid	3,4-dihydroxycinnamic acid	Alkaline	12.2	180.2	0.56	72.2	55.3
<i>p</i> -Coumaric acid	4-hydroxycinnamic acid	Alkaline	13.2	164.2	0.61	39.4	53.1
Vanillin	4-hydroxy, 3-methoxybenzaldehyde	Organic	16.5	152.1	0.66	65.3	85.3
Ferulic acid	4-hydroxy, 3-methoxycinnamic acid	Alkaline	17.8	194.2	0.52	44.4	68.0
Quercetin	3,3',4',5',7'-pentahydroxyflavone	Organic	21.5	302.2	0.33	72.2	85.1
LSD (0.05)						3.5	4.1

^aSee Figure 1 for fractions 1-9.^bThe average lengths of roots and shoots for control were 5.7 and 4.5 mm, respectively.

could be classified into three levels based on concentration. Dilute concentrations (5 mg fresh weight/Petri dish) did not inhibit seed germination and shoot length but root lengths were reduced. With 10 mg fresh weight/Petri dish, root and shoot lengths were affected more than seed germination. Twenty-five milligrams fresh weight per Petri dish involved complete inhibition of seed germination and resulted in blackening of the seeds.

Phenolics that have been isolated and identified from lantana leaves are umbelliferone, methyl coumarin, gentisic acid, β -resorcylic acid, α -resorcylic acid, *p*-hydroxybenzoic acid, salicylic acid, vanillic acid, caffeic acid, *p*-coumaric acid, vanillin, ferulic acid, and quercetin. These compounds have been reported to be allelopathic (McPherson et al., 1971; Rice, 1984; Fay and Duke, 1977).

Umbelliferone, methyl coumarin, vanillin, quercetin, and a few unknown compounds were found in fraction 5. All other identified compounds were found only after acid or alkaline hydrolyses. Gentisic acid was identified in the acid hydrolysate; and caffeic, *p*-coumaric, and ferulic acids were identified in the alkaline hydrolysate. The other phenolic acids were found in the acidic and basic fractions (Table 4). Overall, more phenolic compounds were found in the alkaline extracts.

To verify the phytotoxic effect of these compounds, 100 ppm solutions of individual authentic compounds were tested on ryegrass growth (Table 4). Of the 13 compounds tested, umbelliferone, methyl coumarin, β -resorcylic, *p*-hydroxybenzoic, salicylic, and ferulic acids reduced ryegrass growth. The aromatic acids were generally more active than the alcohols (Table 4). The activity of phenols decreased as the number of hydroxyl groups increased.

We conclude from these studies that several phenolic compounds found in lantana inhibit ryegrass seed germination and seedling growth. The severity of growth inhibition depended on the concentration and compound. Roots were affected more than shoots. Information on naturally occurring plant growth inhibitors may provide leads for the development of new, more efficient and selective herbicides.

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BEHAVIOR AND SURVIVAL OF WESTERN SPRUCE BUDWORM, *Choristoneura occidentalis* FREEMAN, EXPOSED TO AN ω -FLUORINATED PHEROMONE ANALOGUE

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Abstract— ω -Fluorinated (*E*)-11-tetradecenal (14F-E11-14:Al) was synthesized using 1,10-decanediol as a starting compound. Male and female western spruce budworm, *Choristoneura occidentalis* Freeman, moths exposed to 14F-E11-14:Al had LC_{50s} (CI) of 12.0 (7.4–19.5) μ g and 41.7 (22.7–76.2) μ g, respectively. Antennectomized moths had lower mortality rates than normal moths. 14F-E11-14:Al elicited slightly lower amplitudes than 97:3 (*E/Z*)-11-tetradecenal (*E/Z*11-14:Al) in electroantennogram studies, but the lag or recovery period following excitation by 14F-E11-14:Al was much lower than for *E/Z*11-14:Al at the same concentrations. 14F-E11-14:Al and *E*11-14:Al evoked similar responses from male moths in wind-tunnel evaluations but neither compound was as effective as a virgin female in stimulating moths to fly upwind to contact the lure.

Key Words—Fluorinated pheromone analog, western spruce budworm, *Choristoneura occidentalis*, Lepidoptera, Tortricidae, electroantennogram, wind tunnel, toxicity, (*E*)-14-fluorotetradec-11-en-1-ol.

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INTRODUCTION

Fluorine is used as an isosteric replacement for hydrogen in organic molecules, and several fluorinated insect pheromones have been synthesized that show biological activity similar to the natural pheromones (Camps et al., 1984a, b; Briggs et al., 1986). An ω -fluorinated analog of the trail pheromone of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), displayed latent toxicity (Carvalho and Prestwich, 1984) possibly by a mechanism similar to the in vivo β -oxidation of long-chain ω -fluorinated fatty acids to fluoroacetate (Pattison, 1959). Thus the fluorinated analog acted as a proinsecticide, and the oxidative enzymes of the insects were recruited to generate the potent toxin fluoroacetate (Prestwich, 1986; Prestwich et al., 1984), which would block the citric acid cycle by the formation of fluorocitrate (Peters, 1957).

We synthesized the ω -fluorinated analog of the major component of the western spruce budworm, *Choristoneura occidentalis* Freeman, a major defoliator of Douglas-fir in western North America (Furniss and Carolin, 1977), and evaluated its biological activities by toxicity determinations, wind-tunnel studies, and the electroantennogram technique.

METHODS AND MATERIALS

Synthesis

All reagents were obtained from commercial suppliers and were used without further purification. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl, and dichloromethane (CH_2Cl_2) and triethylamine were distilled from CaH_2 . [^1H]NMR spectra were recorded at 270 MHz on a modified Nicolet-Oxford H-270 spectrometer, and at 400 MHz on a Bruker WH-400 spectrometer in deuteriochloroform with tetramethylsilane as the internal standard. EI mass spectra were recorded on a Kratos AEI MS-902 or MS-50 spectrometer at 70 eV. Infrared spectra of the neat liquids were obtained on a Perkin-Elmer 710B instrument. Thin-layer chromatography was performed using Merck (Kieselgel 60F₂₅₄, 0.2 mm) precoated TLC plates. Flash chromatography (Still et al., 1978) was performed on Merck silica gel G (400–230 mesh) using ethyl acetate–petroleum ether, bp 60–80, mixtures (v/v). Elemental analysis was carried out by Mr. P. Borda of the UBC Microanalytical Laboratory. GC analyses were carried out on a Hewlett-Packard 5880A gas chromatograph using an OV-101 glass capillary column (12 m) with a He flow rate of 1.0 ml/min, and a temperature program of 50–260°C at 20°C/min. Structures of compounds numbered below are shown in Figure 1.

2-(9-Bromononyl)-1,3-dioxane (**3**). Dimethyl sulfoxide (35.5 g, 0.5 mol) was added slowly dropwise over a period of 30 min to a cold (Dry Ice–acetone)

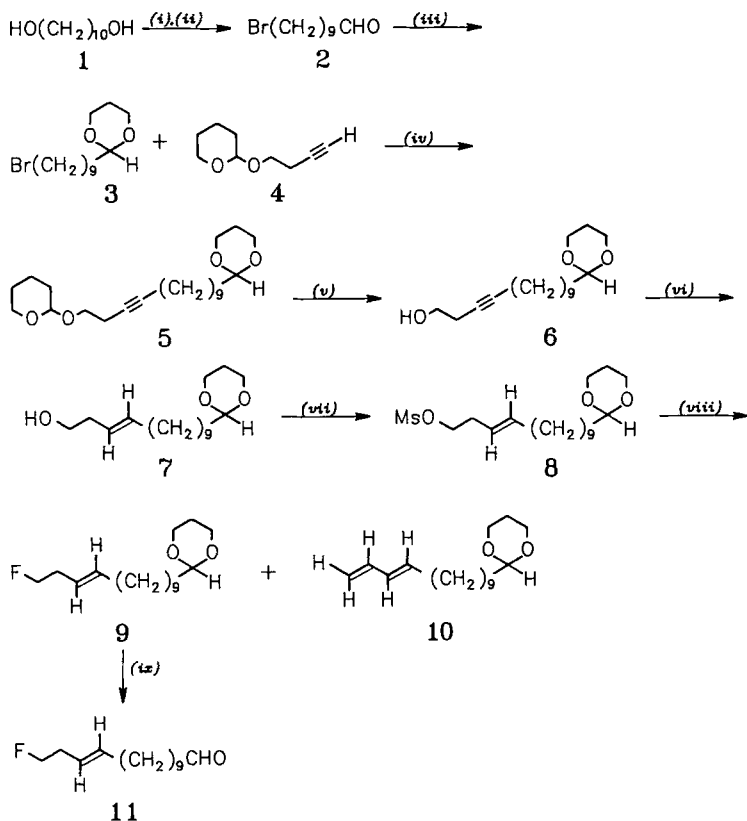


FIG. 1. Scheme for synthesis of (*E*)-14-fluorotetradec-11-en-1-al (**11**). Reagents: (i) 48% HBr, H₂O, heptane, 80°C; (ii) DMSO, (COCl)₂, Et₃N, -78°C; (iii) HO(CH₂)₃ OH, PPTS, PhCH₃, reflux; (iv) LiNH₂, NH₃ liq; (v) PPTS, MeOH; (vi) LiAlH₄, diglyme, 140°C; (vii) CH₃SO₂Cl, Et₃N, CH₂Cl₂, 0°C; (viii) nBu₄NF, THF; (ix) AcOH, H₂O, reflux.

solution of oxalyl chloride (22.1 ml, 0.25 mol) in CH₂Cl₂ (750 ml) stirring under N₂. The solution was stirred for a further 20 min, and then a solution of 10-bromodecanol (50.0 g, 0.21 mol) in CH₂Cl₂ (90 ml) was added slowly dropwise over a period of 45 min. After a further 15 min, triethylamine (100 ml, 0.72 mol) was added dropwise and the mixture was allowed to warm to room temperature over a 3-hr period. The reaction was quenched by addition of water, and the reaction mixture was washed with water, 2 M HCl, and satd NaCl soln. After drying and removing the solvent, the crude product was obtained as a yellow oil (53.7 g). The crude aldehyde, 1,3-propanediol (22.8 ml, 0.32 mol)

and PPTS (1.6 g, 6.4 mmol) were dissolved in toluene (500 ml) in a Dean-Stark apparatus and refluxed for 5.5 hr. The reaction was cooled, washed with water, satd NaHCO_3 soln, and NaCl soln. After drying and removing the solvent, the crude product was subjected to vacuum distillation. A colorless oil distilling at 120–140°C at 0.25 mm Hg was collected (51.8 g, 84%): IR (neat) cm^{-1} 1140 (C—O), 2750, 2657 (—O—CHR—O—); MS m/z (%) 293, 291 ($\text{M}^+ - 1$, 12.52, 13.36), 211 (4.14), 137, 135 (14.67, 14.83), 123, 121 (3.12, 4.70), 115 (10.70), 102 (26.24), 97 (15.75), 95 (18.93), 86 (100); [^1H]NMR δ 1.19–1.44 (m, 13H), 1.44–1.59 (m, 2H), 1.95 (quin, 2H), 2.02 (m, 1H), 3.36 (t, 2H), 3.70 (t, 2H), 4.04 (dd, 2H), 4.45 (t, 1H).

2-{13-[(2'-Tetrahydropyranyl)oxy]tridec-10-ynyl}-1,3-dioxane (5). A solution of lithium amide was prepared by adding lithium metal (0.31 g, 19 mmol) to dry liquid ammonia (100 ml, distilled from sodium) under N_2 , and adding a small quantity (1 mg) of ferric nitrate. A solution of 4 (1.50 g, 9.7 mmol), prepared from but-3-yn-1-ol, in THF (10 ml) was added and the mixture left stirring for 1.25 hr. A solution of 3 (2.78 g, 9.5 mmol) in THF (20 ml) was added dropwise over a period of 10 min. The mixture was allowed to warm to room temperature, and the ammonia was evaporated over a period of 21 hr. The residue was dissolved in EtOAc and washed with water, 0.2 M HCl, satd NaHCO_3 soln, and satd NaCl soln. After drying and removing the solvent, the crude product was placed on a silica gel column (80 g) and eluted with 10% EtOAc–petroleum ether, collecting fractions of 20–25 ml. Fractions 17–28 were shown by GC and TLC to contain the product and were combined and evaporated to obtain a yellowish oil (1.74 g, 50%): IR (neat) cm^{-1} 2750, 2680 (—O—CHR—O—), 1145, 1080, 1035 (C—O); MS m/z (%) 366 (M^+ , 0.13), 87 (84.4), 85 (100); [^1H]NMR δ 1.16–1.57 (m, 21H), 1.64 (tt, 1H), 1.72–1.84 (m, 1H), 1.99–2.10 (m, 1H), 2.05 (m, 2H), 2.38 (m, 2H), 3.40–3.50 (m, 2H), 3.65–3.76 (m, 3H), 3.82 (m, 1H), 4.03 (dd, 2H), 4.44 (t, 1H), 4.58 (t, 1H).

(E)-2-(13-Hydroxytridec-10-enyl)-1,3-dioxane (7). A solution of 5 (5.88 g, 16 mmol) and PPTS (0.44 g, 1.7 mmol) in methanol (50 ml) was stirred under N_2 for 22 hr. The reaction mixture was poured into water, extracted with EtOAc, and the organic layer washed with water, satd NaHCO_3 soln, and satd NaCl soln. After drying and removing the solvent, a yellow oil 6 was obtained (4.74 g). A suspension of lithium aluminum hydride (2.1 g, 55 mmol) in diglyme (50 ml) and THF (10 ml) was heated under N_2 at 110°C for 30 min, during which time a small amount of liquid was distilled from the reaction vessel. The solution was cooled to 0°C and a solution of 6 in diglyme (12 ml) was added via syringe. The solution was then maintained at 140°C for 93 hr. The reaction mixture was then slowly poured into a mixture of ice and satd Rochelle salt soln which was then extracted with EtOAc. The organic layer was washed with water, and satd NaCl soln, dried, and the solvent removed. The crude product was placed on a silica gel column (200 g) and was eluted with 30% EtOAc–

petroleum ether, collecting fractions of 25 ml. Fractions containing the product were combined and evaporated to obtain a yellowish oil (3.32 g, 69.6%): IR (neat) cm^{-1} 3450 (—OH), 1150, 1060 (C—O), 980 (CH=CH); MS m/z (%) 284 (M^+ , 0.17), 283 (1.00), 254 (0.94), 209 (1.55), 87 (100); [^1H]NMR δ 1.20–1.47 (m, 15H), 1.53–1.64 (m, 2H), 2.01 (q, 2H), 1.87–2.18 (m, 1H), 2.26 (q, 2H), 3.63 (t, 2H), 3.76 (td, 2H), 4.11 (dd, 2H), 4.53 (t, 1H), 5.41 (dt, 1H), 5.56 (dt, 1H).

(*E*)-2-(13-Fluorotridec-10-enyl)-1,3-dioxane (**9**). A solution of **7** (1.80 g, 6.3 mmol) in CH_2Cl_2 (50 ml) was cooled to 0°C and triethylamine (1.30 ml, 9.50 mmol) was added, followed 5 min later by methanesulfonyl chloride (0.59 ml, 7.60 mmol). The mixture was stirred for 3 hr, then diluted with dichloromethane and washed with water, 0.3 M HCl, satd NaHCO_3 soln, and satd NaCl soln. After drying and removing the solvent, a yellowish oil was obtained. This crude product was dissolved in THF (50 ml), 4 Å molecular sieves (3.0 g) and Bu_4NF (1 M in THF, 10.0 ml) were added, and the mixture stirred at room temperature under N_2 overnight (19 hr). More Bu_4NF (2.0 ml) was added and the mixture refluxed for 2 hr. The reaction was filtered through a short silica gel plug, which was washed with Et_2O and EtOAc. The filtrate was evaporated, and the residue dissolved in EtOAc and washed with water and satd NaCl soln. After drying and removing the solvent, the crude product was placed on a silica gel column (40 g) and eluted with 5% EtOAc–petroleum ether. Fractions containing the second slower-moving product were combined and evaporated. This crude product and maleic anhydride (0.5 g, 5 mmol) were dissolved in toluene (50 ml) and heated to reflux for 5 hr. The reaction mixture was diluted with EtOAc and washed with water, satd NaHCO_3 soln, and satd NaCl soln. After drying and removing the solvent, the crude product was placed on a silica gel column (40 g) and eluted with 5% EtOAc–petroleum ether, collecting fractions of 10 ml. Those fractions containing the product were combined and evaporated to obtain a yellowish oil (0.76 g, 42%): IR (neat) cm^{-1} 2750, 2690 (—O—CHR—O—), 1150 (C—O), 1000 (C—F), 970 (CH=CH); MS m/z (%) 286 (M^+ , 0.93), 285 (4.15), 266 (1.40), 87 (100); [^1H]NMR δ 1.23–1.42 (m, 15H), 1.53–1.61 (m, 2H), 1.99 (q, 2H), 1.95–2.12 (m, 1H), 2.38 (dq, 2H, $J_{\text{HF}} = 24$ Hz), 3.74 (td, 2H), 4.08 (dd, 2H), 4.34, 4.46 (dt, 2H, $J_{\text{HF}} = 46$ Hz), 4.49 (t, 1H), 5.38 (dt, 1H), 5.54 (quin, 1H). Anal. Calcd. for $\text{C}_{17}\text{H}_{31}\text{FO}_2$: C, 71.28; H, 10.91. Found: C, 70.98; H, 11.04.

(*E*)-14-Fluorotetradec-11-en-1-ol (**11**). A solution of **9** (0.67 g, 2.3 mmol) in acetic acid (9 ml) and water (1.5 ml) was heated at reflux under N_2 for 5 hr. The reaction mixture was diluted with Et_2O and washed with water, satd NaHCO_3 soln, and satd NaCl soln. After drying and removing the solvent, the crude product was placed on a silica gel column (40 g) and eluted with 5% EtOAc–petroleum ether, collecting fractions of 10 ml. The purest fractions were combined and evaporated to yield a colorless oil (0.27 g, 50%), which was

shown to be 97.6% pure by GC. (The only other peak on the GC trace was assigned to the corresponding chloro compound). Combination of the other fractions yielded less pure product (0.14 g, 26%): IR (neat) cm^{-1} 2750 ($-\text{CHO}$), 1730 ($\text{C}=\text{O}$), 1010 ($\text{C}-\text{F}$); MS m/z (%) 210 ($\text{M}^+ - \text{H}_2\text{O}$, 1.51), 209 ($\text{M}^+ - \text{F}$, 0.45), 208 ($\text{M}^+ - \text{HF}$, 2.13), 200 ($\text{M}^+ - \text{C}_2\text{H}_4$, 0.32), 122 (22.04), 121 (24.06), 98 (34.21), 94 (78.84), 55 (100); [^1H]NMR δ 1.20–1.42 (m, 12H), 1.64 (m, 2H), 2.03 (q, 2H), 2.43 (td, 2H), 2.31–2.49 (m, 2H), 4.35, 4.53 (dt, 2H, $J_{\text{HF}} = 48\text{Hz}$), 5.44 (dt, 1H), 5.54 (dt, 1H), 9.93 (t, 1H, $-\text{CHO}$). Anal. Calcd. for $\text{C}_{14}\text{H}_{25}\text{FO}$: C, 73.64; H, 11.04. Found: C, 73.49; H, 11.09. Mol. Wt. Calcd. for $\text{C}_{14}\text{H}_{23}\text{F}$ ($\text{M}-\text{H}_2\text{O}$): 210.1785. Found by high resolution mass spectrometry: 210.1789.

Bioassay

Budworms were reared on artificial diet in 180-ml plastic cups in the laboratory at 24–26°C and 30–50% relative humidity. The moths were maintained in individual 180-ml cups at 16:8 light–dark with scotophase at 1500 hr (PST). Adult emergence was checked daily, except on weekends, and males were used in wind-tunnel experiments when two to six days posteclosion.

Toxicity Tests. LC_{50}s of (*E*)-14-fluorotetradec-11-en-1-al (14F-E11-14:Al) were determined for both male and female western spruce budworm moths. 14F-E11-14:Al was diluted in *n*-heptane at concentrations ranging from 0.03 to 3.0 mg/ml. One drop (approx. 14 μl) of a given concentration was added to a circle of filter paper (Whatman 42; 2.00 cm^2). After allowing the solvent to evaporate, the filter paper was placed inside a 180-ml plastic specimen cup containing four or five moths 2–5 days old (mean age = 3.4 days). Each concentration was tested with 8–10 moths. Moths were kept in a fume hood at 23–26°C and 10:14 light–dark and observed after 24 hr. Both 97:3 (*E/Z*)-11-tetradecenal (*E/Z*11-14:Al) and *n*-heptane were used as controls. Mortality was corrected by Abbott's formula and mortalities of 0% and 100% were excluded from computation of the LC_{50} . The LC_{50}s and 95% confidence intervals (CI) were determined for 24-hr exposure and the log-dosage probit line was tested for goodness of fit by chi-square.

To test the hypothesis that toxicity was acting primarily through adsorption and reaction with antennal receptors, we compared the mortality between antennectomized and normal moths exposed to 14F-E11-14:Al. The antennae were severed as near as possible to the head and the moths exposed to one drop on filter paper of either 2.5 mg/ml 14F-E11-14:Al or *n*-heptane for 24 hr.

Antennal Sensitivity to 14F-E11-14:Al. 14F-E11-14:Al and *E/Z*11-14:Al were formulated in polyvinyl chloride (PVC) (Daterman, 1974) in concentrations of 0.0005 to 0.5% (w/w). Antennal responses were recorded using the electroantennogram technique (EAG) described by Roelofs (1977) with modi-

fications described in Sweeney and McLean (1987). Each of 15 male moths was tested for its response to four concentrations of both 14F-E11-14:Al and E/Z11-14:Al. The concentrations were tested in order of increasing strength; at each concentration the treatment order was decided by the toss of a coin. Each replicate was the mean of five treatment responses corrected by subtracting the mean of three control (blank PVC) responses both preceding and following the treatment. Two variables from the EAG were used to quantify the budworm's response: (1) peak amplitude of antennal depolarization; and (2) the time from initial depolarization to the return to the baseline signal (=lag), i.e., the total width of the signal deflection.

Wind-Tunnel Observations. The wind tunnel was as described by Angerilli and McLean (1984) but was modified by removing the baffles from the intake end, adding activated charcoal filters, and reducing its length by 1.2 m. Male moths were released in groups of four to six on a platform 2 m downwind (windspeed = 40 cm/sec) from a lure consisting of either 14F-E11-14:Al, E11-14:Al, or a virgin female, and were observed for 3 min. All lures were held within fiberglass screen cages about 5 × 5 × 3 cm. The moths were scored for wing-fanning, taking off, locking-on (flying a zigzag course upwind towards the pheromone source), landing at the pheromone source, and displays of copulatory behavior on the lure cage (curling their abdomen to the side while wing fanning).

RESULTS AND DISCUSSION

The ω -fluorinated pheromone was prepared by the sequence of reactions shown in Figure 1. 10-Bromodecanal (**2**) was obtained in two steps from 1,10-decanediol (**1**), protected as its cyclic acetal **3**, and coupled with the protected acetylenic alcohol **4** in moderate yield. Acidic hydrolysis of **5** removed the tetrahydropyranloxy group, leaving the acetal intact, and the resultant β -hydroxyacetylene **6** was reduced stereoselectively (LAH/150°C) (Rossi and Carpita, 1977) to the (*E*)-homoallylic alcohol **7**. Preparation of the mesylate **8**, followed by treatment with tetra-*n*-butylammonium fluoride (Foster et al., 1967) yielded a mixture of the desired β -fluoroalkene **9**, and the diene **10**. The latter could be removed by performing a Diels-Alder reaction on the crude reaction product using maleic anhydride in refluxing toluene, and isolating the unreacted β -fluoroalkene **9** by chromatography. An attempt to introduce fluorine directly by the reaction of diethylaminosulfur trifluoride (Middleton, 1975) with a related homoallylic alcohol yielded exclusively the rearranged α -fluorocyclopropane derivative. Acidic hydrolysis of the acetal protecting group of **9** furnished the fluorinated pheromone **11** in good yield.

Toxicity

No males were killed by exposure to 0.03 mg/ml 14F-E11-14:Al so this treatment, rather than the *n*-heptane control, was used to calculate corrected mortalities (Swaroop et al., 1966). No mortality occurred in the E/Z11-14:Al controls. The log-dosage probit regression of percentage mortality vs. concentration of 14F-E11-14:Al was significant for males ($r^2 = 0.89$) and females ($r^2 = 0.80$) and both fitted a straight line (chi-square, $\alpha = 0.05$). The LC₅₀ (95% CI) was 0.62 (0.39-1.01) mg/ml for males and 2.17 (1.18-3.96) mg/ml for females. By using a mean of 52 drops/ml of *n*-heptane from a Pasteur pipet, these LC₅₀ values (95% CI) translate to 12.0 (7.4-19.5) μg and 41.7 (22.7-76.2) μg of 14F-E11-14:Al on filter paper for males and females, respectively.

When we removed the moths' antennae, the respective mortalities of male and female moths that were exposed to 2.5 mg/ml 14F-E11-14:Al were only 55% and 50%, compared to 90% and 70% for normal moths. The drop in mortality was significant for the antennectomized males but not the females (Fisher's exact probability test, $P \leq 0.05$). There was no mortality of antennectomized moths exposed only to *n*-heptane.

These results demonstrate that 14F-E11-14:Al is lethal to western spruce budworm moths and that its toxicity is greater for males than females. The LC₅₀ of 14F-E11-14:Al was more than three times lower for male moths than for female moths. The difference in toxicity between sexes may have been due to differences in body weights, antennal sensitivity, or both. Male budworm pupae weigh about 40% less than female pupae (unpublished data). Although we do not rule out the possible effect of body weight, we suspect that the greater toxicity in the male moths was associated with greater antennal sensitivity to the analog. According to EAG recordings, the female moth is much less sensitive to E/Z11-14:Al than is the male moth (unpublished data). Adult female spruce budworm, *C. fumiferana* (Clemens), have one third to one half the number of pheromone-sensitive sensilla trichodea as males (Albert and Seabrook, 1973) and are correspondingly less sensitive to E/Z11-14:Al in EAG comparisons (Palaniswamy and Seabrook, 1978; Ross et al., 1979).

Removal of the moths' antennae significantly reduced the percentage mortality of the males but mortality still occurred. This suggests that 14F-E11-14:Al enters the moth not only through the antennae but also via other body surfaces. Lonergan (1986) found a cuticular enzyme in male and female spruce budworm moths that degraded adsorbed aldehyde pheromone to its corresponding acid. Esterases that degrade pheromone adsorbed to body scales and which probably serve to clean the insect of uncontrolled pheromone sources have been found in the giant silk moth, *Antheraea polyphemus* (Cramer) (Vogt and Rid-diford, 1986a), and the cabbage looper, *Trichoplusia ni* (Hübner) (Ferkovich et al., 1982). From our results we hypothesize that a cuticular enzyme similar

to or the same as that described by Loneragan (1986) is also present in the western spruce budworm moth and that it probably transforms adsorbed 14F-E11-14:Al to (*E*)-14-fluoro-11-tetradecenoic acid. The fluorotetradecenoic acid, due to its even number of carbons, would likely be broken down via β -oxidation to yield the latent toxin fluoroacetate (Peters, 1957; Pattison, 1959).

EAG Response

At the same concentration, 14F-E11-14:Al elicited significantly reduced amplitude and lag than did *E/Z*11-14:Al (Table 1). However, the most notable difference in response was the much shorter recovery period or lag following stimulation with 14F-E11-14:Al. The lag after response to 0.5% *E/Z*11-14:Al was about 14 times longer than the lag after response to 0.5% 14F-E11-14:Al in spite of a difference of only 0.33 mV in amplitude (Table 1).

These data show that the budworm antenna is sensitive to 14F-E11-14:Al, but that the EAG shape is quite different from that of *E/Z*11-14:Al. The difference in amplitude might have been due to different release rates of *E/Z*11-14:Al and 14F-E11-14:Al from PVC but this is unlikely, due to the similarity of molecular size and volatility. The presence of 3% *Z*11-14:Al in the *E/Z*11-14:Al lure may also have contributed to the difference in amplitude; future EAG experiments with 14F-E11-14:Al should include an *E*11-14:Al control. Other possibilities include a difference in the binding affinity at the putative acceptor site and/or fewer of these sites that are sensitive to F-ALD.

TABLE 1. ELECTROANTENNOMETER RESPONSES OF WESTERN SPRUCE BUDWORM MALE MOTHS TO FOUR CONCENTRATIONS OF 14F-E11-14:Al AND *E/Z*11-14:Al FORMULATED IN PVC^a

Concentration in PVC (% w/w)	AMP (mV) ^b		LAG (sec) ^b	
	<i>E/Z</i> 11-14:Al	14F-E11-14:Al	<i>E/Z</i> 11-14:Al	14F-E11-14:Al
0.5	1.34a	1.03b	5.71a	0.41b
0.05	1.06a	0.92b	1.91a	0.13b
0.005	0.65a	0.49b	0.68a	0.10b
0.0005	0.25a	0.16a	0.16a	0.05b

^aEach of 15 male moths was tested for its response to all concentrations of 14F-E11-14:Al and *E/Z*11-14:Al. The concentrations were tested in order of increasing strength; at each concentration the treatment order was decided by the toss of a coin. For each replicate, amplitude (AMP) and LAG responses are the mean of five responses corrected by subtracting the mean of three control responses both preceding and following the treatment.

^bMeans within variable and within row followed by different letter are significantly different; Wilcoxon's matched-pair rank-sum test ($\alpha = 0.05$).

The short recovery period after response to 14F-*E*11-14:Al is very interesting, but we can only speculate on its cause. Roelofs and Comeau (1971) hypothesized that EAG shape and recovery period were related to the affinity of the pheromone component with the putative receptor site, whereas Baker and Roelofs (1976) suggested that differences in EAG recovery rate in response to various compounds might be due to the activation of olfactory neurons with different intrinsic recovery rates. Kaissling (1986) suggested that a long recovery period following a receptor potential might be due to active pheromone molecules remaining at the receptor sites after stimulation and may reflect processes of odor inactivation. The very short recovery period following stimulation by 14F-*E*11-14:Al might be due to an increased affinity for the enzyme(s) responsible for pheromone inactivation, possibly resulting from the substitution of the more electronegative ω -fluorine for the hydrogen. It may also be related to an increased rate of adsorption and transport due to enhanced lipid solubility of fluorinated analogs (Filler and Naqvi, 1982). Experiments using radiolabels, like those of Prestwich et al. (1986) and Vogt and Riddiford (1986b), are required to further elucidate the olfactory biochemistry of the western spruce budworm moth in response to 14F-*E*11-14:Al and *E/Z*11-14:Al.

Behavioral Response to 14F-E11-14:Al

The proportion of budworm that took off, locked-on, reached the lure, and displayed copulatory behavior was not significantly different in response to either 14F-*E*11-14:Al or *E*11-14:Al. However, neither synthetic lure was as effective as a virgin female in eliciting locking-on and upwind flight to the lure (Table 2). This was probably due to the absence of components in the 14F-*E*11-14:Al and *E*11-14:Al lures that are normally present in the virgin female effluvia, chiefly *Z*11-14:Ald. The effluvia of western spruce budworm female moths is composed of a blend of 92:8 *E/Z*11-14:Al + 89:11 (*E/Z*)-11-tetradecenyl acetate + 85:15 (*E/Z*)-11-tetradecenol in about a 10:3:6 ratio (Silk et al., 1982; Cory et al., 1982). Trap catch of western spruce budworm male moths was four times higher in traps baited with 92:8 *E/Z*11-14:Al than in traps baited with the same concentration of *E*11-14:Al alone (Cory et al., 1982). In wind-tunnel experiments with spruce budworm moths, Sanders (1984) found that 95:5 *E/Z*11-14:Al (0.05% in PVC) induced 75% of males to lock-on and sustain flight for greater than 1 min compared to only 10% in response to *E*11-14:Al. We suggest that the addition of *Z*11-14:Al to the 14F-*E*11-14:Al, at the natural ratio (92:8 *E/Z*), would increase the lure's attraction without significantly affecting its toxicity. This remains to be tested however.

Prestwich (1987) described the use of fluorinated pheromone analogs in which the aldehyde moiety has been altered in order to cause sensory disruption. By ω -fluorination, in contrast, we have left the aldehyde functional group intact

TABLE 2. RESPONSES OF WESTERN SPRUCE BUDWORM ADULT MALES TO 0.05% 14F-E11-14:Al (IN PVC), 0.05% E11-14:Al (IN PVC), OR VIRGIN FEMALE IN WIND TUNNEL

Treatment	n (N) ^a	Percentage Response ^b				
		Wing-fan	Take off	Lock-on	Land	Cop ^c
14F-E11-14:Al	27 (31)	77a	85a	48a	33a	15a
E11-14:Al	25 (25)	80a	88a	40a	32a	28ab
Virgin female	29 (31)	87a	86a	83b	55b*	38b

^an = number of moths able to fly. All moths (N) were used to calculate the percentage of moths wing-fanning and displaying preflight copulatory behavior but only fliers (n) were used to calculate the percentage taking off, locking-on, and landing at the source, and the percentage displaying postflight copulatory behavior.

^bPercentages within each column followed by different letters are significantly different. Fischer exact probability test ($\alpha \leq 0.05$ except where * denotes $\alpha \leq 0.10$).

^cCop = copulatory behavior.

and we have produced an analog that retains considerable biological activity as well as being toxic to budworm moths. Sanders (1982) suggested that mating disruption of spruce budworm would be more effective using a few potent pheromone sources rather than numerous low-potency sources and that disruption would be enhanced by adding an insecticide or sterilant to the pheromone. Our results indicate that 14F-E11-14:Al is toxic to the western spruce budworm and is equivalent to E11-14:Al in terms of long-range attraction of males and that it therefore shows potential as a mating disruptant. However, the practical testing of 14F-E11-14:Al as a mating disruptant must await testing of its toxicity to vertebrates and other nontarget organisms. Other even-numbered ω -fluorinated aldehydes have been shown to be highly toxic to vertebrates (Pattison, 1959). Nonetheless, we are optimistic that these hazards may be reduced significantly by the use of low concentrations, low release rates, and by the containment of lures in traps.

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PHEROMONE EMISSION AND BLEND PERCENTAGES IN *Eoreuma loftini*¹ DETERMINED BY TWO METHODS

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Abstract—Calling behavior and pheromone emission by virgin female *E. loftini* moths were investigated in laboratory experiments. Calling peaked during the last three hours of the night. Three-day-old moths called more than older or younger moths and began calling earlier in the night than younger moths. Female emissions were collected in flasks without airflow and cylinders with airflow. Control tests indicated that the pheromone components (Z)-11-hexadecenyl acetate (HDA) and (Z)-13-octadecenylacetate (ODA) were 69 and 54% adsorbed on moths, respectively, and the component (Z)-13-octadecenal (ODL) was 92–99% adsorbed depending on its concentration, when put into flasks with noncalling females for 4 hr. Pheromone exposed to moths for less than 4 hr was adsorbed less. After correction for adsorption, the pheromone blend from females calling in flasks was 9:42:49% of HDA/ODL/ODA with an overall emission rate of 58 ng/female/4 hr. Three-day-old females emitted more pheromone than 0- to 2- or 4- to 5-day-old moths, in flasks. Little or no pheromone put into cylinders either downwind or upwind from a male moth was adsorbed by the moth. The pheromone blends from females calling in cylinders, corrected using downwind and upwind control test results, respectively, were 15:35:50 and 13:40:48% of HDA/ODL/ODA with overall emission rates of 32 and 35 ng/female/night.

Key Words—Sex pheromones, pheromone adsorption, age, pheromone emission, Lepidoptera, Pyralidae, Crambinae, *Eoreuma loftini*, calling behavior, (Z)-11-hexadecenyl acetate, (Z)-13-octadecenyl acetate, (Z)-13-octadecenal.

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INTRODUCTION

Three components of the sex pheromone of *Eoreuma loftini* (Dyar) have been identified by Shaver et al. (1988). The three chemicals, (Z)-11-hexadecenyl acetate (HDA), (Z)-13-octadecenal (ODL), and (Z)-13-octadecenyl acetate (ODA), proved active as sexual stimulants and attractants in laboratory and field experiments when tested in ratios found in abdomens of calling females. However, amounts in abdomens do not necessarily reflect blends emitted by the moths (Tumlinson et al., 1982). In particular, aldehydes are frequently emitted in greater blend percentages than are present in glands (Baker et al., 1980; Ramaswamy and Cardé, 1984). Our work reports when female *E. loftini* of various ages call and how much of each component they emit, as determined by two methods.

METHODS AND MATERIALS

General. Pupae were from cultures that originated in the Lower Rio Grande Valley (LRGV) of Texas. Insects were maintained on a 14:10 light-dark cycle similar to natural summer conditions in the LRGV. Laboratory temperatures ranged between 20 and 27°C and relative humidity between 40 and 75%. Ecdysis began after three to five days under these conditions. Female moths of each age were held in separate wood-frame, screened cages (20 cm/side). Cages were sprayed with water each day during the light period.

Calling Behavior Observations. The numbers of "calling" virgin females of various ages were recorded every half hour during the last 7 hr of the night using night-vision goggles. Calling behavior was described by Brown et al. (1988). Ages were 0, 1, 2, 3, and 4-5 days posteclosion. Since most moths eclosed at night, 0 days posteclosion indicates moths that emerged earlier on the test night. Between 62 and 122 moths of each age were observed over three test nights.

Pheromone Emission in Closed Flasks without Airflow. We collected pheromone emitted by females of various ages in flasks with no airflow using a method similar to that of Baker et al. (1980). Ages were 0, 1, 2, 3, 4, and 5 days posteclosion. For each replication, 25 virgin females of one age were put into a 500-ml round-bottom flask with a glass stopper during the last 4 hr of the night ($N = 10$ replications/age). At the onset of the light period, the flask was chilled at 0°C for 1 h to freeze the pheromone onto the sides of the flask. The dead moths were then discarded from the cold flask. The flask was extracted by shaking for 5 min with each of two 20-ml portions of pentane. The portions were combined, filtered, and concentrated to 100 μ l under a stream of nitrogen with low heat.

To control for sample-handling losses, 100 ng of each component were added to empty 500-ml flasks 4 hr before extraction ($N = 40$). To control for pheromone losses due to adsorption on moths, three additional experiments were conducted. In the first experiment, 0.4, 0.6, and 0.6 μg of HDA, ODL, and ODA, respectively, and 25 males were put into 500-ml flasks 4 hr before extraction ($N = 6$). In the second experiment, various amounts of the three pheromone components and 25 noncalling females were put into flasks 4 hr before extraction. Noncalling females were virgin females used during the photophase. Since these moths moved little compared to calling females, flasks were shaken slightly each hour to stimulate activity. Amounts of HDA, ODL, and ODA, respectively, added to flasks were: 0, 0, and 0 μg ($N = 2$); 0.1, 0.15, and 0.15 μg ($N = 3$); 0.4, 0.6, and 0.6 μg ($N = 8$); 0.7, 1.0, and 1.0 μg ($N = 5$); 1.0, 1.5, and 1.5 μg ($N = 3$); 1.3, 2.0, and 2.0 μg ($N = 6$); and 2.0, 3.0, and 3.0 μg ($N = 3$). In the third experiment, 25 noncalling females were put into flasks and 0.4, 0.6, and 0.6 μg of the three components were introduced in four equal increments at 4, 3, 2, and 1 hr before extraction ($N = 8$). This experiment was to measure adsorption when pheromone was introduced in a way more like calling females would emit it. Extractions of flasks in control experiments were performed exactly as in tests with calling females.

Pheromone Emission in Cylinders with Airflow. This method was used to minimize adsorption of pheromone on calling females. Females of ages 0, 1, 2, 3, 4, and 5 days posteclosion were tested ($N = 2/\text{age}$). For each replication, one virgin female was put into a vertical cylinder (20 cm long \times 0.85 cm ID) with its abdomen oriented downward. The tapered bottom of the cylinder contained glass wool covered with 20 mg of Porapak Q (Supelco, Inc., Bellefonte, Pennsylvania) (50/80 mesh). A 5-cm section containing the moth was located 10 cm above the Porapak Q and was delineated above and below by plugs of glass wool. Another 50 mg of Porapak Q covered the glass wool over the moth section. A humidified airflow of ca. 2–5 cm/sec was directed downward through the cylinder. Moths remained in the cylinders for the entire 10-hr scotophase, except 0-day-old moths, which were put into the cylinders for the last 4 hr of the night.

Cylinders containing the bottom 20 mg of Porapak Q were cleaned with successive rinses of 100 ml of acetone, 100 ml of 10% acetone in pentane, and 50 ml of pentane before testing. To extract pheromone, the cylinder, the bottom 20 mg of Porapak Q, and all glass wool downwind from the moth were rinsed with 20 ml of 5% acetone in pentane. The extract was concentrated to ca. 200 μl under nitrogen at low heat.

Several control experiments were conducted. First, 40, 60, and 60 ng of HDA, ODL, and ODA, respectively, were introduced onto the walls of a cylinder 1 cm downwind of a male moth 4 hr before extraction ($N = 2$). Second, 40, 60, and 60 ng were introduced on the glass wool upwind from a male moth

4 hr before extraction ($N = 9$). The second experiment was also conducted with no moth in the cylinder to control for adsorption onto the upwind glass wool ($N = 2$). Finally, the experiment was conducted with a male moth present but no pheromone introduced to the cylinder ($N = 4$). Extractions were performed exactly as with cylinders containing calling females.

Chemical Analyses. Extracts were prepared for gas chromatography (GLC) using a Waters liquid chromatograph (HPLC) (Waters Associates, Milford, Massachusetts) to remove hydrocarbons that interfere with detection of the pheromones. Two methods were used, as dictated by availability of materials. For flasks with calling females and flask controls without moths, the extract was injected onto a NOVA-PAK C18 Radial-PAK HPLC cartridge (4 μm particle size) in an RCM-100 module using a mobile phase of acetonitrile at 2 ml/min. Detection was at 205 nm (model 490 Programmable Multiwavelength Detector). Eluent fractions were collected from 2 ml of elution volume before the retention volumes of the three pheromones until 2 ml of elution volume after the pheromone retention volumes, as determined by standards. The k' values were ca. 4, 4, and 7 for ODL, HDA, and ODA, respectively. The fractions were combined and reduced to 5–10 μl under a stream of nitrogen at low heat. Gas chromatography was conducted using a Shimadzu GC-9A instrument (Shimadzu Scientific Instruments Inc., Columbia, Maryland) with a flame ionization detector. Analyses were on an SPB-5 capillary column (Supelco) (60 m, fused silica, 0.32 mm ID, 1 μm film). Helium carrier gas linear velocity was 25 cm/sec. A splitless injection (0.5 min split valve closed) of 1–2 μl was used. The column oven conditions were: 50°C for 5 min; 10°C/min increase until 250°C; 250°C isothermal.

For all other pheromone emission and control experiments, extracts were injected onto a Resolve silica Radial-PAK HPLC cartridge (5 μm particle size) in an RCM-100 module. The mobile phase was 2 ml/min of the following: hexane for 2 min; 0.5% isopropyl alcohol in hexane for 4 min; and 1% isopropyl alcohol in hexane for 14 min. Eluent was collected from 18–30 ml of elution volume since ODL eluted between 20–24 ml and HDA and ODA eluted between 26–28 ml. The fraction was concentrated to 5–7 μl , all of which was injected onto a DB-225 Megabore column (J & W Scientific, Rancho Cordova, California) (30 m, fused silica, 0.53 mm ID, 1 μm film). Helium carrier gas linear velocity was 60 cm/sec. A splitless injection was used as before. The column oven conditions were: 50°C for 5 min; 10°C/min increase until 170°C; 2°C/min increase until 190°C; 190°C isothermal. The two chemistry methods proved identical by tests with standards.

Pheromone amounts were quantitated by comparing peak heights to those of standards. Standard HDA and ODL (Sigma Chemical Company, St. Louis Missouri) were greater than 95% pure. Standard ODA (Bedoukian Research, Danbury, Connecticut) was 99% pure. Known amounts of (*Z*)-11-tetradecenyl

acetate and methyl arachidonate were added to the syringe and coinjected with pheromone standards and extracts. Elution of (*Z*)-11-tetradecenyl acetate preceded the earliest-eluting pheromone component, and elution of methyl arachidonate followed the latest-eluting pheromone. The two purposes for these chemicals were to correct for retention-time shifts and response-factor changes over the course of numerous analyses with varying injection volumes.

Statistical Analyses. The calling experiment was a randomized complete block design with three replications of a two-way classification of five age and 15 time treatments. Proportions of calling moths were transformed to arcsins of the square roots for analysis of variance. Age, time, and age/time interactions were included in the model. Means and groups of means were compared by *t* tests and single-degree-of-freedom *F* contrasts, respectively, using the error term from the analysis of variance.

The flask pheromone-collection experiment was completely randomized. Analyses of variance tested effects of age on total emission (HDA + ODL + ODA) and on blend percentages, both uncorrected for adsorption on moths.

Flask control experiments were completely randomized. For tests with noncalling females in which pheromone was introduced 4 hr before extraction, analyses of variance tested effects of amounts added to flasks on the percent recoveries of the three components. The treatment effect was partitioned into linear regression and deviations. Effects were tested using the experimental error term.

Estimations of variances of derived results calculated by multiplication and/or division of two or more means were conducted by addition of relative variances (Skoog and West, 1969).

RESULTS AND DISCUSSION

Calling Behavior. Few females called during the first half of the 10-hr night according to preliminary observations. Most calling occurred during the last 3 hr of the night (Figure 1). In general, older moths began calling earlier. This was demonstrated by a large age/time interaction ($P < 0.001$) resulting from earlier rises in the curves of the older moths. Kanno (1979) and Das and Islam (1982) also found that older *Pyralidae* females called earlier than younger moths.

More older moths called at most times during the peak calling period. More 1-day-old than 0-day-old moths, more 2-day-old than 1-day-old moths, and more 3- and 4- to 5-day-old than 2-day-old moths called ($P < 0.01$). An exception was that fewer 4- to 5-day-old moths than 3-day-old moths called during the peak hours ($P < 0.01$), possibly due to senescence (Tamaki, 1985). Similar effects of age on maximum calling rate have been observed in other

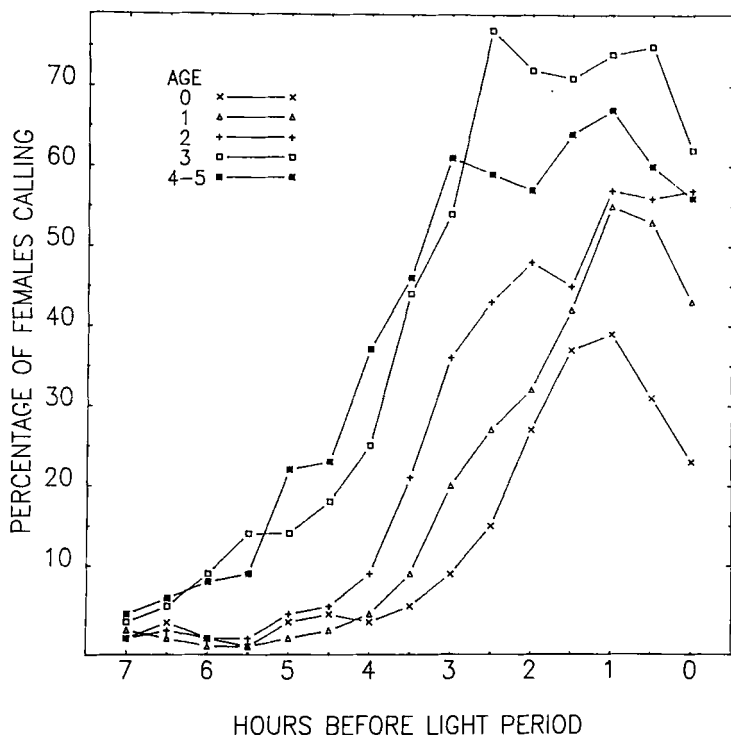


FIG. 1. Calling behavior of virgin female *Eoreuma loftini* moths of different ages.

Pyralidae (Kanno and Sato, 1978; Kanno, 1979; Das and Islam, 1982; Hirai, 1982).

Calling behavior declined just before the beginning of the light period (0 hr = 2-3 min before the lights came on) (Figure 1). Significant decreases occurred during the last hour for 0-, 1-, 3-, and 4- to 5-day-old moths ($P < 0.05$). This suggests that cessation, as well as onset, of calling behavior is a circadian rhythm rather than simply a direct response to light.

Pheromone Recovery in Flask Control Experiments. Percent recoveries of pheromone components from flasks without moths were: HDA, 49.9%; ODL, 42.1%; and ODA, 49.5% (Table 1). Nearly all losses occurred during the final concentrating procedure and syringe loading before GLC analysis.

Pheromone amounts extracted from flasks with noncalling females to which no pheromone was added were: HDA, 0.4 ng/25 females; ODL, 0.3 ng; and ODA, 5.0 ng. It is important that the amount of ODL extracted from these flasks was low since experiments discussed below showed that ODL recovery

TABLE 1. PERCENT RECOVERIES OF PHEROMONE FROM FLASK CONTROL EXPERIMENTS COMPARING PRESENCE OF MOTHS, SEX, AND TIMING OF PHEROMONE INTRODUCTION

	Percent recoveries (SE) ^a		
	HDA	ODL	ODA
Without moths ^b	49.9 (3.2) d	42.1 (3.0) c	49.5 (3.3) c
With males ^c	9.6 (1.5) a	0.33 (0.07) a	15.8 (2.2) a
With noncalling females ^c	14.9 (1.2) b	0.56 (0.11) ab	24.4 (2.0) b
Noncalling females, increments ^d	20.0 (2.0) c	1.56 (0.51) b	29.2 (3.0) b

^aHDA = (Z)-11-hexadecenyl acetate; ODL = (Z)-13-octadecenal; ODA = (Z)-13-octadecenyl acetate. Means in the same vertical column followed by the same letter are not significantly different at the 5% level by *t* tests.

^b0.1 μg each of HDA, ODL, ODA introduced 4 hr before extraction.

^c0.4 μg HDA, 0.6 μg ODL, 0.6 μg ODA, introduced 4 hr before extraction.

^d0.4 μg HDA, 0.6 μg ODL, 0.6 μg ODA, introduced in four increments at 4, 3, 2, and 1 hr before extraction.

rates depend on amounts in flasks. The extracted 0.3 ng ODL translates into 0.016 μg in the flask before losses, according to methods discussed in a later section. This amount is small relative to amounts of ODL introduced to flasks (0.15–3.0 μg) and probably little affected recovery rates.

Unknown compounds with retention times within 0.1–0.3 min of the pheromone peaks were also found in flasks with noncalling females to which no pheromone was added. These unknowns interfered with pheromone quantitation. Therefore, to correct data from tests in which pheromone was added to flasks containing noncalling females, we subtracted the following amounts from those recovered: HDA, 1.4 ng/25 females; ODL, 1.8 ng; and ODA, 8.1 ng. These amounts include pheromone from noncalling females and the unknown peaks.

Recoveries from flasks containing 0.4 μg HDA, 0.6 μg ODL, 0.6 μg ODA and moths were lower than recoveries from flasks without moths (*P* < 0.05) (Table 1), indicating adsorption onto moths that were discarded before flask extraction. Pheromone recoveries from flasks containing males, to which pheromone was introduced 4 hr before extraction, were: HDA, 9.6%; ODL, 0.33%; and ODA, 15.8%. Recoveries from flasks containing noncalling females, to which pheromone was also introduced 4 hr before extraction, were: HDA, 14.9%; ODL, 0.56%; and ODA, 24.4%. Male/female differences in recoveries (HDA, ODA: *P* < 0.05; ODL: *P* = 0.10) may be related to behavior as males were more active during tests. Recoveries from flasks containing noncalling

females to which pheromone was introduced in four increments were: HDA, 20.0%; ODL, 1.56%; and ODA, 29.2%. These recoveries were higher than from flasks with noncalling females to which all pheromone was added 4 hr before extraction (HDA, $P < 0.05$; ODL, $P = 0.08$; ODA, $P = 0.21$). Pheromone introduced at 1, 2, and 3 hr before extraction apparently did not have as much time to adsorb on moths as that added 4 hr before extraction. The greater the adsorption affinity, the more the incremental technique increased recovery rate. Thus, recovery of ODL increased more (178%) than recoveries of HDA (34%) and ODA (20%). Since the incremental technique is similar to pheromone emission by females, these results suggest recoveries from flasks with calling females should also be 20.0%, 1.56%, and 29.2% for HDA, ODL, and ODA, respectively. Note, however, that these values are accurate only for HDA and ODA. Recovery of ODL depends on the amount of ODL in flasks ($0.6 \mu\text{g}$ in this test) as reported below.

Flask experiments with noncalling females and various amounts of pheromone added 4 hr before extraction indicated that percent recovery was independent of amount added for HDA and ODA (regression coefficients were not significant) but not for ODL. Mean recoveries of HDA and ODA, over all amounts added to flasks, were ca. 16 and 23%, respectively. Regression of percent recovery of ODL on amounts added to flasks gave the equation:

$$\text{Recovery (\%)} = 1.38 (\text{amount added to flask}) - 0.17$$

The coefficient, 1.38, was significant ($P < 0.05$). The correlation coefficient, r , was 0.46. Linear regression (1 df) accounted for 84% of the treatment effect (5 df), indicating a good fit to linearity. The low r was due to variability associated with the greater amounts of ODL introduced to flasks (1.5, 2.0, 3.0 μg). Recovery rates ranged from 0.2 to 10.1% for these treatments compared to 0.1–1.8% for the three lower treatments.

Estimation of Percent Adsorption on Moths. Percent adsorptions were estimated for flask control experiments with noncalling females in which various amounts of pheromone were added 4 hr before extraction (Table 2). Percent adsorption was calculated by dividing the percent recovery from flasks with moths by the percent recovery from flasks without moths, subtracting the quotient from 1, and multiplying by 100. This calculation assumed that handling losses from flasks without moths does not depend on amounts added to flasks. Table 2 shows that HDA and ODA adsorption rates on moths did not vary with amounts of HDA and ODA added, but ODL adsorption increased with decreasing amounts of ODL added to flasks ($P < 0.05$ by regression). Mean adsorptions were: HDA, 69.0%; ODL, 96.2%; and ODA, 54.1%. Similar adsorption trends were reported for *Grapholitha molesta* (Lepidoptera: Tortricidae) with two components of its pheromone (Baker et al., 1980).

Effects of Age on Pheromone Emission by Females in Closed Flasks. Blend

TABLE 2. PERCENT ADSORPTION OF PHEROMONE BY NONCALLING FEMALE MOTHS IN FLASK CONTROL EXPERIMENTS WITH VARIOUS AMOUNTS OF INTRODUCED PHEROMONE

Amount introduced to flask (µg)	Percent adsorbed (SE) ^a		
	HDA	ODL	ODA
0.10	71.5 (4.4) ab	—	—
0.15	—	99.4 (0.2) a	56.0 (6.0) a
0.40	70.2 (2.4) ab	—	—
0.60	—	98.7 (0.3) a	50.7 (4.0) a
0.70	77.6 (3.2) b	—	—
1.0	72.3 (4.4) ab	98.6 (0.7) a	59.7 (5.5) a
1.3	58.8 (6.4) a	—	—
1.5	—	93.6 (5.0) a	60.0 (4.6) a
2.0	66.4 (7.2) ab	92.6 (3.7) a	48.8 (5.6) a
3.0	—	92.2 (5.9) a	52.7 (7.7) a
Overall Mean	69.0 (2.1)	96.2 (1.1)	54.1 (2.2)

^aHDA = (Z)-11-hexadecenyl acetate; ODL = (Z)-13-octadecenal; ODA = (Z)-13-octadecenyl acetate. Means in the same vertical column followed by the same letter are not significantly different at the 5% level by LSD.

percentages of the three components, corrected for handling losses but not for adsorption on moths, changed with moth age (Table 3). Apparent blends became richer in ODA and poorer in HDA and ODL each day as females aged from 0 to 3 days ($P < 0.05$), then remained constant with further aging. We believe

TABLE 3. PHEROMONE BLENDS EXTRACTED FROM FLASKS CONTAINING 25 FEMALE *Eoreuma lofini* MOTHS OF VARIOUS AGES, UNCORRECTED FOR ADSORPTION ON MOTHS

Age (days)	Percentage of blend (SE) ^{a,b}			Pheromone/female/4 hr (ng) (SE) ^b
	HDA	ODL	ODA	
0	14.3 (0.9) a	9.2 (2.7) a	76.5 (3.4) a	7.6 (1.3) a
1	14.5 (0.9) a	7.0 (1.2) ab	78.4 (1.6) ab	15.9 (2.5) ab
2	12.0 (0.7) b	5.7 (1.6) ab	82.4 (2.1) bc	22.4 (3.0) b
3	8.6 (0.4) c	3.7 (0.5) b	87.7 (0.7) c	34.9 (5.5) c
4	8.9 (0.3) c	3.3 (0.6) b	87.8 (0.7) c	21.6 (2.8) b
5	7.4 (0.5) c	4.7 (1.5) ab	87.9 (1.5) c	16.3 (2.9) ab

^aHDA = (Z)-11-hexadecenyl acetate; ODL = (Z)-13-octadecenal; ODA = (Z)-13-octadecenyl acetate.

^bMeans in the same vertical column followed by the same letter are not significantly different at the 5% level by LSD.

the change in blend with age is an experimental artifact. Figure 1 showed that most calling by 0-day-old females occurred during the last 2 hr of the night, whereas calling by 3- to 5-day-old females was evenly spread over the last 3 hr. Calling by 1- to 2-day-old females was intermediate. Applying the results from the incremental pheromone introduction test, pheromone from younger moths had less time to adsorb than that from older moths. Therefore, percent recoveries of HDA and ODL from younger moths should be higher than their recoveries from older moths. Recovery of ODA should follow a similar trend, but to a lesser extent since it adsorbs less on moths. Thus, it was adsorptions that changed with age; blend percentages only appeared to change as a result of changes in recoveries.

Table 3 also shows total pheromone (HDA + ODL + ODA), per female, extracted from flasks with calling females. The data were corrected for handling losses but not for adsorption. Three-day-old females emitted the most pheromone ($P < 0.05$) and newly emerged females the least ($P < 0.05$). However, females either did not call continuously or not all females called during the last 4 hr of the night (Figure 1). Dividing pheromone per female per 4 hr in Table 3 by the mean percentages of females calling during the last 4 hr of the night as estimated from Figure 1 gives an approximate correction for noncalling time or noncalling females. Thus, pheromone emissions per calling female per 4 hr, uncorrected for adsorption, were: 0 days old, 36 ng; 1 day old, 50 ng; 2 days old, 55 ng; 3 days old, 56 ng; and 4 to 5 days old, 36 ng. Correction for adsorption at each age would require data that we do not have concerning adsorption per unit time.

Calculation of Pheromone Blend in Closed Flasks with Calling Females.

Assuming that effects of age on pheromone emission were averageable, corrected blend percentages and emission rates were calculated from extracted amounts of HDA, ODL, and ODA without regard to age. Mean amounts extracted per female were: HDA, 1.08 ng (SE = 0.005 ng); ODL, 0.46 ng (0.002 ng); and ODA, 8.25 ng (0.051 ng).

To correct the HDA and ODA emission data for adsorption and handling losses, the mean extracted amounts were divided by their respective recovery rates from the incremental-introduction tests (Table 1). Thus the amount of HDA emitted per female per 4 hr was $1.08/0.200 = 5.4$ ng. The amount of ODA per female per 4 hr was $8.25/0.292 = 28.3$ ng.

For the ODL emission data, corrections were made for incremental introduction and for regression. First, the recovery rate for 0.6 μg of ODL introduced in increments was divided by the recovery rate for 0.6 μg of ODL introduced 4 hr before extraction (Table 1). The ratio was $1.56\%/0.56\% = 2.79$. This ratio was used to correct the regression equation assuming that the

effect of introducing ODL in increments is constant over all amounts of ODL tested:

$$\text{Recovery (\%)} = 2.79 [1.38 (\text{amount in flask}) - 0.17]$$

Since percent recovery equals the amount extracted (amt. ext.) divided by the amount in the flask (amt. flask), times 100, the equation was rewritten as:

$$\text{amt. ext.} = 2.79 [1.38 (\text{amt. flask})^2 - 0.17 (\text{amt. flask})]/100$$

This equation was used to correct the extracted amount of ODL for changes in percent recovery as amounts of ODL in flasks changed. For 11.5 ng extracted per flask (0.46 ng/female × 25 females/flask), the mean amount in the flasks was 612 ng/flask or 24.5 ng per female. Since 612 ng is close to the 0.6 μg used in determination of the ratio 2.79, error due to nonconstancy of the ratio with changing amounts of ODL is minimal.

Adding amounts of HDA, ODL, and ODA yields total pheromone emission, corrected for adsorption and handling losses, of ca. 58 ng per female in flasks per 4 hr (Table 4). Correcting for the mean percentage of females calling for all ages over the last 4 hr of the night yields an emission rate of 126 ng per calling female per 4 hr. Note, however, that females that do not appear to be calling may be emitting some pheromone. Blend percentages were ca.: HDA, 9%; ODL, 42%; and ODA, 49% (Table 4).

Pheromone Recovery in Cylinder Control Experiments. No peaks were found at pheromone retention times when no pheromone was added to cylinders

TABLE 4. PHEROMONE BLENDS FROM VIRGIN FEMALE *Eoreuma loftini* AS DETERMINED IN CLOSED FLASKS AND IN CYLINDERS

	Percentage of blend ^a (SE)			Total pheromone/female (ng) (SE) ^b
	HDA	ODL	ODA	
Closed flasks	9 (1)	42 (12)	49 (5)	58 (12)
Cylinder				
Downwind	15 (2)	35 (6)	50 (11)	32 (4)
Control				
Cylinder				
Upwind	13 (2)	40 (10)	48 (11)	35 (5)
Control				

^aHDA = (Z)-11-hexadecenyl acetate; ODL = (Z)-13-octadecenal; ODA = (Z)-13-octadecenyl acetate.

^b4-hr collection time for flasks; 10-hr collection time for cylinders.

with male moths. However, unknown peaks were found within 0.1–0.3 min of pheromone retention times. To correct data from tests in which pheromone was added to cylinders and tests with calling females, we subtracted the following amounts from those recovered: HDA, 0.15 ng; ODL, 0.80 ng; and ODA, 1.25 ng.

When pheromone was introduced in the cylinder downwind from a male moth, recoveries were: HDA, 41.1%; ODL, 36.8%; and ODA, 38.6% (Table 5). Losses may have been caused by inability to extract all pheromone from cylinders, trap breakthrough, postextraction sample handling, and oxidative breakdown. Since recoveries were only about 5–11% less than those in flasks without moths (Table 1), most losses from cylinders were probably due to postextraction sample handling.

When pheromone was introduced on the glass wool upwind from a male moth, recoveries were: HDA, 20.6%; ODL, 10.6%; and ODA, 13.0%. These recoveries do not take into account adsorption to the upwind glass wool which was not solvent-extracted.

Adsorbances to the upwind glass wool were estimated as: HDA, 52.6%; ODL, 63.3%; and ODA, 64.5%. These were calculated as follows: Recoveries from cylinders in which pheromone was introduced on the upwind glass wool with no moth present (HDA, 19.5%, ODL, 13.5%, ODA, 13.7%) were divided by recoveries from cylinders in which pheromone was introduced downwind from a male moth (41.1%, 36.8%, 38.6%) to correct for losses due to sample handling, etc. Quotients were: HDA, 47.4%; ODL, 36.7%; and ODA, 35.5%. These are estimates of pheromone evaporation from the upwind glass wool. Adsorptions were found by subtracting these percentages from 100%.

TABLE 5. PERCENT RECOVERIES OF PHEROMONE FROM CYLINDER CONTROL EXPERIMENTS IN WHICH PHEROMONE WAS INTRODUCED DOWNWIND OR UPWIND FROM MALE MOTH

	Percent recoveries (SE) ^a		
	HDA	ODL	ODA
Downwind	41.1 (2.8) a	36.8 (2.2) a	38.6 (6.5) a
Upwind ^b	43.5 (4.6) a	28.9 (5.3) a	36.6 (7.1) a
Difference	-2.4 (5.4)	7.9 (5.7)	2.0 (9.6)

^aHDA = (Z)-11-hexadecenyl acetate; ODL = (Z)-13-octadecenal; ODA = (Z)-13-octadecenyl acetate. Means in the same vertical column followed by the same letter are not significantly different at the 5% level by t-tests.

^bOnly pheromone not adsorbed to the upwind glass wool was used in the calculations.

Recoveries from cylinders in which pheromone was introduced on the glass wool upwind from a male moth, corrected for adsorption to the glass wool, were: HDA, 43.5%; ODL, 28.9%; and ODA, 36.6% (Table 5). These were calculated by dividing the uncorrected recoveries (20.6%, 10.6%, 13.0%) by percentages evaporated from the glass wool (47.4%, 36.7%, 35.5%).

Subtracting "upwind" recoveries from "downwind" recoveries (Table 5) yields estimates of pheromone adsorptions on the male moth in the "upwind" experiment: HDA, -2.4%; ODL, 7.9%; and ODA, 2.0%. None of these differences is statistically significant. However, the larger difference for ODL is consistent with its strong adsorption on moths in flask experiments (Table 2). Possibly, some ODL adsorbed on the male as it passed over his body and/or adsorbed on scales that had fallen onto the downwind glass wool.

Pheromone Emission by Females in Cylinders. Although not statistically significant, 3-day-old females again appeared to emit the most pheromone. Age did not affect blend percentages of the uncorrected data.

To correct for adsorption and handling losses, data from all ages were combined. Mean amounts extracted per female, uncorrected for losses, were: HDA, 1.96 ng (SE = 0.20 ng); ODL, 4.04 ng (0.76); and ODA, 6.16 ng (0.90). The data were corrected using the results of both cylinder control experiments. In each case, the mean extracted amounts were divided by their respective recovery rates from Table 5. For the downwind control test, the corrected emission data per female per night were: HDA, 4.8 ng; ODL, 11.0 ng; and ODA, 16.0 ng. Total pheromone emission (HDA + ODL + ODA) per female per night was 32 ng (Table 4). Blend percentages were: HDA, 15%; ODL, 35%; and ODA, 50% (Table 4). For the upwind control test, the corrected emission data per female per night were: HDA, 4.5 ng; ODL, 14.0 ng; and ODA, 16.8 ng. Total pheromone per female per night was 35 ng (Table 4). Blend percentages were: HDA, 13%; ODL, 40%; and ODA, 48% (Table 4).

How much emitted pheromone that blew back over the bodies of females calling in cylinders is not known. Therefore, we do not know which control test is most applicable. Most likely, turbulence caused some emitted pheromone to blow back over the females. Also scales that had fallen from the female onto the downwind glass wool may have adsorbed some pheromone. Thus the most applicable correction factors may lie between those from the two control tests.

Pheromone Emission vs. Gland Extracts. Amounts of pheromone in glands are not necessarily the same as the amounts and blend percentages females emit (Tumlinson et al., 1982; Mayer and Mankin, 1985). In particular, aldehydes are often more prevalent in emissions than in gland extracts (Baker et al., 1980; Ramaswamy and Cardé, 1984). Our work shows that the sex pheromone of *E. loftini* fits this trend. We report a blend of ca. 9–15%, 35–42%, and 48–50% for HDA, ODL, and ODA, respectively, from calling females. Shaver et al.

(1988) reported a blend of ca. 10:13:78 for these three components extracted from glands of females of this species. Further, we found total pheromone emission (HDA + ODL + ODA) of ca. 32–58 ng per female per night, while Shaver et al. (1988) found only ca. 30 ng in glands. This latter result suggests that pheromone biosynthesis is concurrent with emission (Nordlund and Brady, 1974) such that the amount in the gland at any time is only a portion of what is produced during a day.

Comparison of Methods. This work used two methods for collecting pheromone emitted by calling moths. The cylinder method with airflow proved the simpler, more direct method. Its principal advantage was that there was little or no adsorption on moths, resulting in higher recoveries and less reliance on control test correction factors. Another advantage was that pheromone was emitted in moving air, a more natural situation (Kaae and Shorey, 1972). However, the flask method may have advantages over cylinders when pheromone emission is low and many emitters are needed to trap a quantifiable amount of pheromone.

Females in flasks without airflow produced more pheromone than females in cylinders with airflow ($P < 0.05$ by t test) (Table 4). This is the opposite result from that obtained by Ramaswamy and Cardé (1984) with *Chloristoneura fumiferana* (Lepidoptera: Tortricidae). We have no explanation for the discrepancy, although a group stimulation effect in flasks is possible.

Because of differential adsorption of pheromone components as demonstrated here and by Baker et al. (1980), it now seems critical that collection methods do not allow pheromone emitted by one female to pass over other females, even in moving air. It would be difficult indeed to control for this effect in a chamber containing 100 calling females.

Baker et al. (1981) reported efficient trapping of pheromone on glass wool without the need for chemical adsorbents. Their method eliminates the contamination that accompanies use of chemical trapping agents. Recoveries from their apparatus were 90–100%. Recoveries from our cylinders, after adjusting for postextraction losses of 50–60%, were also about 90–100%. The method of Baker et al. (1981) also employed forced extrusion of the female's pheromone gland, so that adsorption onto scales was minimized, thus minimizing correction for adsorption. Our cylinder method allows females to call naturally and also requires little correction for adsorption onto scales (Table 5). By trapping with glass wool, as reported by Baker et al. (1981), our cylinder method would have been substantially improved to allow clean, efficient collection of pheromone emitted by naturally calling females. Unfortunately, we were unaware of the work of Baker et al. (1981) until after our work was completed.

Finally, an intriguing question with regard to adsorption is: How much pheromone emitted by a female moth is adsorbed on her body and on her immediate environment in nature? Perhaps optimal blends for male response are the

result not only of what the female emits but also of differential adsorption of components by nearby leaves and by the female oriented in a characteristic way in an airstream.

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EFFECT OF MELIACEOUS SEED EXTRACTS ON GROWTH AND SURVIVAL OF *Spodoptera frugiperda* (J.E. SMITH)¹

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Abstract—Hexane and ethanol extracts of seeds from 10 plant species (including neem—*Azadirachta indica* A. Juss.) of the family Meliaceae were incorporated into artificial diet at various doses and fed to fall armyworm [*Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae)] larvae in no-choice tests. All produced significant mortality, reduced larval growth rate, increased time to pupation, or all three, at some concentration. The two highest doses of all of the ethanol extracts caused 100% mortality before pupation, but the hexane extracts tended to be less effective. *Aglai cordata* Hiern. ethanol extract was as potent as the comparable neem seed extract at virtually all levels, and its hexane extract was active at much lower concentrations than the neem extract was. The sublethal effects (slower growth and increased time to pupation) were usually detectable at lower doses of extract than mortality was.

Key Words—*Spodoptera frugiperda*, Lepidoptera, Noctuidae, Meliaceae, neem, insecticides, limonoids, seed extracts.

INTRODUCTION

Interest in the possible application of natural products to insect pest management continues at a high level. Numerous research programs are engaged in searching for and developing plant-derived products that produce deleterious effects in insects. Although this research encompasses many plant families and classes of compounds (Abivardi and Benz, 1984; Lane et al., 1985; Saxena et

¹ Mention of firm or product names does not imply recommendation or endorsement by USDA over others not mentioned.

al., 1986; Villani et al., 1985), the most widespread effort is currently being applied to limonoids, primarily from meliaceous plants (Chiu, 1985; Hassanali et al., 1986; Prabhaker et al., 1986; Siddiqui et al., 1986).

The complexity of many limonoids may preclude economically feasible syntheses; this complexity is exemplified by the structure of azadirachtin (the insecticidal compound from neem), which has recently been revised (Kraus et al., 1985; Broughton et al., 1986) 10 years after it was proposed by Zanno et al. (1975). Because synthesis of azadirachtin is unlikely, and its isolation in pure form is difficult and tedious, most agricultural applications will probably utilize extracts that have undergone some degree of fractionation to concentrate the active components.

Plants of the family Meliaceae are widely distributed in tropical and subtropical areas where they might be available for pest control in local agriculture. We previously investigated the feeding deterrence and mortality produced by seed extracts from 22 meliaceous species on fall armyworm (FAW) larvae [*Spodoptera frugiperda* (J.E. Smith)] and on striped cucumber beetle adults [*Acalymma vittatum* (F.)] (Mikolajczak and Reed, 1987). These mortality data were obtained at a single dose level (10,000 ppm) in artificial diet, and, at this level, a number of the extracts were as active as comparable neem seed extracts. We therefore conducted this study (an expansion of our earlier work) of the growth-regulating properties of some of these extracts on FAW larvae using doses of 10,000 ppm and lower.

METHODS AND MATERIALS

Extracts. The names of plant species investigated appear in Table 1. Seeds were identified and collected by USDA botanists, Beltsville, Maryland. Hexane and ethanol extracts were prepared as previously described (Mikolajczak and Reed, 1987).

FAW Bioassay. Pinto bean diet as described by Shorey and Hale (1965) was used for rearing FAW larvae and also for the bioassays. Seed extracts were incorporated into the artificial diet in appropriate quantities and the bioassays were set up according to the procedure presented earlier (Mikolajczak and Reed, 1987); the concentrations used were 10,000, 2000, 400, 80, and 16 ppm. All concentrations of each seed's extracts were assayed simultaneously along with a hexane and an ethanol solvent control and a diet blank. Neonate larvae were caged individually in 1-oz plastic cups; 10 replicates were done. The larvae were maintained in darkness at 26–28°C and 55–65% relative humidity. The larvae were weighed on days 7 and 13, and a record was made of those that died prior to pupation. In addition, the number of days required for pupation was also recorded.

TABLE 1. FALL ARMYWORM BIOASSAY RESULTS FOR MELIACEAE SEED EXTRACTS.

Plant name	Hexane extract (ppm)					Ethanol extract (ppm)					
	10,000	2000	400	80	16	10,000	2000	400	80	16	Control
<i>Aglaia cordata</i> Hiem.											
Mortality (%) ^c	NT ^b	100**** ^c	100****	100****	100**** ^d	100****	100****	100****	100****	65**** ^d	23
Larval wt. (% of control) ^e	NT	<1****	<1****	-/	5**** ^d	-	-	-	-	10**** ^d	(157) ^e
Days to pupation	NT	-	-	-	-	-	-	-	-	22.6**** ^d	17.6
<i>Azadirachta indica</i> A. Juss.											
Mortality (%)	100****	80****	10	0	0	100****	100****	100****	100****	100**** ^d	7
Larval wt. (% of control)	-	12****	78	113	108	-	-	-	-	-	(321)
Days to pupation	-	24.5****	17.8	17.0	17.1	-	-	-	-	-	17.4
<i>Chickrassia tabularis</i> A. Juss.											
Mortality (%)	100****	0	10	0	10	100****	100****	50*	0	0	3
Larval wt. (% of control)	-	70**	102	109	107	-	-	5***	98	103	(303)
Days to pupation	-	17.7*	16.7	15.8	16.4	-	-	28.0***	16.7	16.2	16.7
<i>Dysoxylum malabaricum</i> Bedd. ex C. DC.											
Mortality (%)	20	20	10	10	0	100****	100****	10	20	40	13
Larval wt. (% of control)	39****	95	98	99	99	<1****	5***	57****	91	72**	(319)
Days to pupation	22.3****	19.5**	18.0	17.6	18.5	-	-	20.4****	18.0	18.3	17.8
<i>Dysoxylum spectabile</i> (Forst. F.) Hook F.											
Mortality (%)	0	0	10	10	0	100****	100****	30*	0	10	7
Larval wt. (% of control)	18**** ^b	102 ^b	102 ^b	113 ^b	137 ^b	<1**** ^d	5**** ^d	54**** ^d	95 ^d	120 ^d	(146)
Days to pupation	26.2****	18.7	18.3	17.7	18.3	-	-	24.7****	20.3	18.7	19.0
<i>Lansium domesticum</i> Corr.											
Mortality (%)	NT	30	0	0	10	100****	100****	10	10	40	20
Larval wt. (% of control)	NT	44****	97	74	96	-	<1****	35****	89	88	(192)
Days to pupation	NT	24.4****	19.8	20.8	19.0	-	-	23.3****	21.0	20.7	20.3

TABLE 1. Continued

Plant name	Hexane extract (ppm)				Ethanol extract (ppm)					
	10,000	2000	400	16	10,000	2000	400	80	16	Control
<i>Melia acedarach</i> L.										
Mortality (%)	50*	10	40	20	100***	100***	80***	0	10	13
Larval wt. (% of control)	106	98	101	110	88	-	2***	58***	101	(334)
Days to pupation	16.6	16.8	17.2	16.8	17.0	-	32.0***	18.9***	17.1	17.2
<i>Sandoricum koetjape</i> (Burm. F.) Merr.										
Mortality (%)	70***	10	20	30	100***	100***	30	30	20	10
Larval wt. (% of control)	13***	86	92	72	97	<1***	8***	44***	57**	(333)
Days to pupation	24.0***	17.3	17.4	16.1	16.3	-	26.0***	21.0***	21.8***	17.8
<i>Swietenia mahoganii</i> (L.) Jacq.										
Mortality (%)	20	0	0	0	10	100***	40**	0	0	3
Larval wt. (% of control)	41***	102	97	114	102	-	8***	83	105	(283)
Days to pupation	21.3***	16.6	17.2	16.3	17.0	-	26.5***	19.0*	17.5	17.7
<i>Trichilia roka</i> (Forsk.) Chiov.										
Mortality (%)	100***	40	10	10	0	100***	100***	10	0	17
Larval wt. (% of control)	-	79	80	80	83	-	<1***	25***	102	(199)
Days to pupation	-	18.8	20.3	20.2	19.9	-	-	24.7***	20.8	20.1

^aLarval mortality.

^bNT = not tested.

^c*, **, and *** denote significance at the 0.05, 0.01, and 0.001 levels, respectively.

^dMean of two data sets.

^eDetermined on day 13.

^fDash indicates all larvae died prior to recording of data.

^gControl mean weight, mg, shown in parentheses.

^hHexane control used for comparison.

ⁱEthanol control used for comparison.

Treatment of Data. The larval mortality data were analyzed as contingency tables, using chi-square tests. For each experiment, the diet blank and solvent controls were first tested for consistency. Significant differences among controls could indicate incomplete removal of solvents during diet preparation, which would confound conclusions about the effectiveness of extracts. No differences among controls were detected, however (the probability levels for the chi-square statistics were >0.05 in all cases), and the data for diet blank, hexane, and ethanol controls were pooled. Each experimental treatment was then tested against the combined controls. Extracts were considered active when the probability level for the chi-square statistic was <0.05 .

Sublethal effects, as evidenced by lower larval weights at 7 and 13 days, and increased times to pupation, were detected by one-way analysis of variance. Time to pupation was analyzed in the original scale, but a variance-stabilizing transformation was required for the larval weight data. Conversion to the logarithmic scale was satisfactory. Upon finding a significant overall F statistic in an analysis, each extract treatment was then compared to the combined controls (diet blank and solvent controls) using a t test. As with the mortality data, consistency of controls was checked before pooling the control data. In the few cases where differences among controls were detected, treatments were compared to the appropriate solvent control only.

RESULTS AND DISCUSSION

Table 1 presents the test data for concentrations of extracts from 10,000 ppm to 16 ppm in the artificial diet. To conserve test materials during this early stage of the project, tests were limited to 10 replications. *A. cordata* and *Lansium domesticum* hexane extracts were not tested at the highest concentration due to scarcity of material. In some cases, the mortality figure was 100% prior to pupation, but a value is also listed for larval weight; in these cases the larvae that were weighed on day 13 subsequently died before pupation. This effect can be seen (Table 1) in the higher ethanol extract concentrations for the *Dysoxylum* species, *Sandoricum koetjape*, and others, and also for three of the *A. cordata* hexane concentrations. Otherwise, a dash in the larval weight row indicates all larvae died before day 13.

Overall, the effects produced by the ethanol extracts were more pronounced than those resulting from the hexane extracts. The ethanol extracts of all species caused 100% larval mortality at doses of 2000 ppm and above. Extracts of two species, neem and *A. cordata*, had considerably lower activity thresholds, the former causing 100% mortality at 16 ppm, and the latter at 80 ppm. The ethanol extract of *Trichilia roka* was intermediate in activity, with 400 ppm causing 100% mortality. Although the ethanol extracts of all the spe-

cies were active, only that of *A. cordata* produced activity approaching that of neem ethanol extract, which contains most of the azadirachtin found in the seed. Differences in the activities of these extracts could be due to the presence of different deleterious compounds, quantitative differences in these compounds, or both. Further chemical purification would be required to resolve qualitative and quantitative effects. It is likely that inert compounds account for much of the extracts' weights.

Ethanol extracts of *L. domesticum* and *Melia azedarach* produced 100% mortality at 10,000 ppm (Table 1), but in our earlier work (Mikolajczak and Reed, 1987), neither caused any mortality at the same dose. We feel that this difference may be due, in large part, to the age difference between the two groups of test insects—6-day-old larvae in the previous experiments as opposed to neonate larvae in the current experiments.

The hexane extract of *A. cordata* was considerably more active than those of the other species, including neem, causing 100% mortality at only 16 ppm. Only three other hexane extracts caused 100% mortality, and then only at the highest concentration tested (Table 1). The ability of hexane to extract appreciable activity from *A. cordata*, but not from neem, suggests that the active compound(s) in *A. cordata* are less polar than azadirachtin.

The 7- and 13-day larval weights, and time before pupation were examined for evidence of sublethal effects. There were highly significant differences among the 13-day weights (Table 1). Larval weight was a more sensitive indicator of extract activity than mortality. In 13 cases, a significantly slower weight gain was detected in the absence of significant mortality. This usually occurred one dose level below where mortality became significant. However, significant mortality always corresponded to significantly smaller larvae at day 13, with one marginal exception (the hexane extract of *Melia azedarach* at 10,000 ppm). The significantly lower weights for larvae fed the *Dysoxylum malabaricum* and *Sandoricum koetjape* ethanol extracts at 16 ppm were unusual, since effects were seen at three dose levels below where mortality became significant.

The 7- and 13-day larval weights led to the same conclusions about these extracts; for the sake of clarity we present only the 13-day data in the table. In 78 of 80 treatments in which larvae remained alive through day 13, weights differed significantly from controls at day 13 but only when they also differed at day 7. When expressed as a percentage of control weights, the 7-day and 13-day mean weights were strongly correlated ($r = 0.86$).

Time to pupation showed highly significant differences among extracts (Table 1). It was negatively correlated with the 13-day larval weights: the smaller the larvae at day 13, the longer it took them to pupate. A linear regression model was fitted using the 18 treatments in which both time to pupation and 13-day larval weight were significantly different from control levels. For

larval weights between 75% and 5% of the control value, mean time to pupation increased by ca. 1.3 days for each decrease in weight of 10 percentage points (slope significant, $P \ll 0.001$, $r^2 = 0.67$). For larval weights above ca. 75% of the control value, neither the larval weight nor time to pupation tended to differ significantly from the control. Mean larval weights that were less than 5% of the control weights on day 13 usually led to 100% mortality before pupation.

In cases where an extract caused lower larval weights at day 13, surviving larvae required a longer development time, but pupae tended to be of normal weight (data not shown). Thus, although the larvae seemingly overcame the adverse effects of the diet treatments, their longer development time might benefit IPM strategy by reducing the number of generations per season.

SUMMARY

The mortality-producing capabilities of ethanol extracts of the seeds from nine species of meliaceous plants at 10,000 and 2000 ppm are equivalent to neem ethanol extract, which contains the insecticidal compound, azadirachtin. Hexane extracts of three species, *A. cordata*, *Chickrassia tabularis*, and *T. roka*, caused the same amount of mortality as neem seed hexane extract did at 10,000 ppm; however, *A. cordata* hexane extract proved superior to neem hexane extract at all other doses.

These data, plus our earlier antifeedant results (Mikolajczak and Reed, 1987), clearly demonstrate that extracts of many meliaceous plants could be used for pest control in much the same way neem extracts are. Additionally, we have demonstrated the existence of specific activities different from those displayed by neem extracts. Some examples are the high activity of *A. cordata* hexane extract at very low levels, described here, and the minimal feeding deterrence of *C. tabularis* hexane extract coupled with high mortality and the potent feeding deterrence of *Swietenia mahogani* hexane extract coupled with virtually no mortality, described earlier. Perhaps certain of these plants will find utility in areas where they are native as simple pest-control formulations requiring little expense or equipment to prepare.

Although limonoids may be responsible for much of the observed activities, some cases of non-azadirachtin-like activity could be due to other classes of simpler compounds, which might be more amenable to synthesis. We will continue to pursue the identification of active components from selected meliaceous species as additional plant material is obtained.

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BEHAVIOR AND SURVIVAL OF *Reticulitermes hesperus* BANKS (ISOPTERA: RHINOTERMITIDAE) ON SELECTED SAWDUSTS AND WOOD EXTRACTS

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Abstract—Survival of *Reticulitermes hesperus* workers was assessed in *Pseudotsuga menziesii*, *Lysiloma seemanii*, and *Tabebuia ochracea* sawdusts; and on heartwood solvent extracts of *P. menziesii*, *L. seemanii*, *T. ochracea*, *Pinus ponderosa*, *Tabebuia guayacan*, and a *Centrolobium* species. Survival in *P. menziesii* sawdust was 100% at 5 days and 81–87% at 15 days. Survival in *L. seemanii* and *T. ochracea* sawdusts was significantly less over both 5 and 15 days than in the starvation control, indicating toxicity. Survival on filter papers treated with solvent extracts of *T. ochracea* and *P. ponderosa* was significantly less than that on control papers, but only *P. ponderosa* differed significantly from the starvation control. In behavioral assays with groups and with individual *R. hesperus* workers, extracts of *P. menziesii* and *P. ponderosa* were preferred. In the individual behavioral assays, extracts of *T. guayacan* and *T. ochracea* were repellent. Results of toxicity assays were not predictable from preference assays.

Key Words—*Reticulitermes hesperus*, Isoptera, Rhinotermitidae, termite feeding behavior, termite bioassay, tropical hardwoods, wood extractives.

INTRODUCTION

Subterranean termites in the genus *Reticulitermes* (Isoptera: Rhinotermitidae) are important structural pests throughout North America. Along the Pacific coast, *Reticulitermes hesperus* Banks is the most destructive species (Weesner, 1965; Ebeling, 1975). Currently, use of preservative-treated wood and soil treatment with insecticides are the principal methods used to prevent or control subterranean termite damage.

Naturally occurring toxicants and semiochemicals (e.g., attractants, arrestants, repellents, feeding deterrents, and feeding stimulants) may offer safer alternatives to the current reliance upon insecticidal treatments. Extractives from termite-resistant woods have been under investigation for many years (Wolcott, 1924) and offer some promise as protective treatments for nonresistant woods (Carter and Beal, 1982). However, Smythe and Carter (1970) demonstrated that different termite species may respond differently to sawdust from the same tree species, requiring that all target termite species be investigated. Despite the economic importance of *R. hesperus*, the feeding responses of this species to different woods have not previously been studied. In addition, there has been little evaluation of behavioral (as opposed to toxic) effects associated with termite-resistant woods. As with feeding responses, chemical stimuli may elicit quite different behavioral responses in different termite species (Becker et al., 1972).

In the present study, investigations were conducted on the survival and behavior of *R. hesperus* workers on wood of a North American conifer readily fed upon by termites, and wood of five tree species thought likely to possess some resistance to termite feeding. Bioassays were designed to examine the relative importance of behavioral and toxicological factors in termite feeding and survival on these species. The behavioral responses of both groups and individual *R. hesperus* workers were determined.

Woods of six tree species were evaluated. Two of these, *Pseudotsuga menziesii* (Mirb.) Franco and *Pinus ponderosa* Dougl. ex Laws., are softwoods (Family Pinaceae) of North American origin. The other four species are Central American hardwoods: *Centrolobium* sp. (Leguminosae), *Lysiloma seemanii* Britt. & Rose (Leguminosae, subfamily Mimosaceae), *Tabebuia guayacan* (Seem.) Hemsl. (Bignoniaceae), and *Tabebuia ochracea* (Cham.) Standl.

P. menziesii and *P. ponderosa* are, respectively, the first and second most important timber-producing tree species in North America (Harlow et al., 1979). *P. menziesii* is used extensively for house construction in California, and the three termite colonies used in our experiments were collected from Douglas-fir lumber. In studies with other termite species (Carter, 1976; Carter and Smythe, 1974; Smythe and Carter, 1970), *P. menziesii* heartwood and sapwood have been found to promote termite feeding and survival. These same studies have shown *P. ponderosa* heartwood to be detrimental to termite feeding and survival.

One of the four tropical hardwood species, *T. guayacan*, resists subterranean termite attack in field tests with heartwood stakes (Bultman and Southwell, 1976) and has been found to reduce survival of the termites *Coptotermes formosanus* Shiraki and *Reticulitermes flavipes* (Kollar) (Beal et al., 1974), as have congeners of the other hardwood species (Beal et al., 1974; Wolcott, 1950). Selection of these four particular tropical hardwoods from the great number of

possibly termite-resistant species was due in part to convenience. The heartwoods of *T. guayacan* and *Centrolobium* sp. were already under chemical investigation (Jurd and Wong, 1984), and solvent extracts were available for biological assays. The other two tree species, *L. seemanii* and *T. ochracea*, are abundant in Guanacaste Province, Costa Rica, and were collected there in conjunction with other studies (Frankie et al., 1983). These species were selected before their collection by correlating an extensive study of Costa Rican tree phenology (Frankie et al., 1974) with the available literature on termite resistance, especially Beal et al. (1974), Carter (1976), and Wolcott (1950). Their abundance in Guanacaste Province suggests resistance to the structural damage caused by termites feeding on the heartwood of living trees, a common occurrence in the tropics (Sen-Sarma, 1986). Although not harvested commercially, these woods are locally reputed to be termite and decay resistant (GWF, unpublished observation).

In termite-infested buildings, differential feeding by termites on apparently identical timbers is frequently observed by researchers and pest-control personnel (Kofoid and Bove, 1934). To examine whether *R. hesperus* workers responded differently to adjacent structural timbers exhibiting different amounts of feeding, we included sawdust from field-collected *P. menziesii* timbers in the survival assays.

METHODS AND MATERIALS

Source of Insects. Western subterranean termites, *R. hesperus*, were collected from three sites in Alameda County, California. One colony (Berkeley) was removed from a Douglas-fir, *P. menziesii*, form board embedded in the soil beside a residential driveway in the city of Berkeley, the second (Gilman) from infested Douglas-fir wall framing in a residence in Berkeley, and the third (Oakland) from infested Douglas-fir floor joists in a residence in the city of Oakland. Termites were removed from the wood and kept in separate groups in plastic trays at room temperature (21–25°C) in a humidity chamber (94 ± 5% relative humidity) (Grace, 1986). Only workers (pseudergates, or undifferentiated individuals older than the third instar as determined by size), were used in assays due to their predominance in foraging activities.

Preparation of Sawdusts and Wood Extracts. Solvent extracts of the heartwood of *P. menziesii*, *P. ponderosa*, *T. guayacan*, and *Centrolobium* sp. were provided by L. Jurd and G.D. Manners (Western Regional Research Center, USDA, ARS, Albany, California). These woods were purchased from local merchants in California, and the *Centrolobium* species could not be identified to species by the United States Forest Products Laboratory in Madison, Wisconsin (L. Jurd, personal communication). Dried concentrates from five-day

continuous acetone extraction in a Soxhlet apparatus were diluted 1 g/10 ml acetone. Due to crystalline insolubility, *P. menziesii* extract was diluted in equal parts CH₃OH:CHCl₃.

L. seemanii and *T. ochracea* heartwoods, collected in Costa Rica, were ground in a Wiley mill (40-mesh screen), and 200 g of each sawdust was extracted by soaking in methanol at room temperature (21–25°C) for seven days. The resulting filtrates were concentrated by rotary evaporation and desiccation under vacuum, yielding ca. 10 g *L. seemanii* and 17 g *T. ochracea* dry extracts, which were diluted 1 g/10 ml methanol.

Survival in Douglas-Fir Sawdust. Samples of the timbers from which the *P. menziesii* solvent extracts were prepared were not available, and a separate experiment was designed with two field-collected *P. menziesii* samples. These samples were from two adjacent floor joists in a severely infested house where the Oakland colony was collected. One of these timbers had been extensively tunneled by termites, while the other exhibited only minor tunneling. Excluding the tunneled areas, such sample (heartwood plus sapwood) was reduced to sawdust in a Wiley mill (40-mesh screen).

The assay consisted of an open, 40-mm × 27-mm bottom diam. × 37-mm top diam., ca. 30-ml (1 US fluid oz., Anchor Hocking No. 5175) plastic cup containing 2 g of sawdust, 2 ml steam-distilled water, and 40 *R. hesperus* workers from the Oakland colony. These cups were placed in a humidity chamber, and termite mortality recorded by gently removing and replacing the contents of each cup at 5 and 45 days. This assay was replicated six times with each of the two *P. menziesii* samples.

Survival in Hardwood Sawdusts. Of the six woods used in this study, only samples of the unextracted heartwood of *L. seemanii* and *T. ochracea* were available, since the others were provided in the form of solvent extracts. These two species were reduced to sawdust and assayed as described above for *P. menziesii* sawdust, except that mortality of Oakland workers was recorded at 5, 15, and 45 days. Each substrate was tested with three groups of 40 termites. Alpha-cellulose (Alphacel, Nutritional Biochemicals Corp., Cleveland, Ohio) was included as a feeding control. A starvation control with 2 g white sand in lieu of sawdust was performed under the same experimental conditions, although not simultaneously with the other substrates.

Survival on Wood Extracts. Survival on filter papers treated with solvent extracts of the six woods was assayed in a manner comparable to that used with sawdusts. A 25-mm-diam. Whatman No. 1 filter paper disk was saturated with 100 μl of wood extract (diluted 1 g/10 ml solvent). Each feeding substrate thus consisted of ca. 18% wood extractives by weight. Treated disks were air-dried ca. 20 min, and placed in an open 30-ml plastic cup with 40 Oakland workers. The cups were placed in a humidity chamber and termite mortality recorded at 5, 15, and 45 days. Each wood extract was tested with three groups of termites.

Starvation (no filter paper) and feeding (filter papers treated with methanol only) controls were included in the experiment.

Group Behavioral Responses. Preference (either attraction or arrestment) or repulsion of groups of 10 *R. hesperus* workers (Berkeley and Gilman colonies) in response to wood extracts was measured in an assay similar to that used by Amburgey and Smythe (1977) to assess termite responses to solvent extracts of fungus-decayed wood. A 9-cm-diam. Whatman No. 1 filter paper disk treated with 1 ml wood extract (sufficient to saturate the disk), equivalent to ca. 15% extractives by weight, and air-dried ca. 20 min was cut in half and each half taped to one half of a solvent-treated paper disk. Each reconstituted two-choice disk was placed in an open glass Petri dish, uniformly illuminated by overhead fluorescent lighting (13.5–19.5 foot-candles) at room temperature (21–24°C). Five *R. hesperus* workers were gently deposited onto each half of the disk. The number of termites (in the group of 10) on the extract-treated half of the disk was recorded every 30 sec for 20 min.

The behavioral response of the group was calculated as the number of times more than five insects were recorded on the extract-treated half-disk, divided by the number of times more than five insects were recorded on either half-disk. This index of preference thus indicated the proportion of time spent on the extract-treated half-disk by the majority of each group of 10 termite workers. Each assay was replicated eight times, and the mean proportional response computed for each wood extract. Control assays using half-disks treated only with methanol were also performed.

Individual Behavioral Responses. Behavior of individual workers (Gilman and Oakland colonies) was measured similarly to group behavior. An 18-mm-diam. Whatman No. 1 filter paper disk was saturated with 40 μ l wood extract (ca. 15% extractives by weight), air-dried ca. 20 min, and paired with a solvent-treated disk in an open 5-cm-diam. glass Petri dish under uniform overhead lighting. A single *R. hesperus* worker was placed on the glass between the two disks, and its position on either (or neither) disk recorded every 30 sec for 20 min.

The behavioral response of each individual was calculated as the number of times it was recorded on the extract-treated disk divided by the number of times it was recorded on either disk. Again, this index of preference indicated the proportion of time spent on the extract-treated disk by the worker. A mean proportional response was computed from 50 individual assays with each wood extract. As in the group assays, control assays with two disks treated only with solvent (methanol) were also performed.

Statistical Analyses. Termite survivorship was analyzed with standard *t* test and analysis of variance (ANOVA) techniques, and means were compared with the Ryan-Einot-Gabriel-Welsch (REGW) multiple *F* Test ($\alpha \leq 0.05$) (SAS Institute, 1982). In the behavioral assays, the mean proportional responses of

groups and of individual termites to wood extracts were tested against the mean response of 0.50 expected under the null hypothesis of no effect (two-tail t test, $\alpha \leq 0.05$) (Dixon and Massey, 1983).

RESULTS AND DISCUSSION

Survival in Douglas-Fir Sawdust. Termite survivorship over 45 days was uniformly high and not significantly different (t test, $P = 0.06$) in sawdust from *P. menziesii* samples exhibiting either minor ($81 \pm 6\%$ surviving) or extensive ($87 \pm 4\%$ surviving) prior tunneling by the same *R. hesperus* colony. Survivorship at five days was 100% for workers in both treatments. These data do not support the hypothesis that the pattern of selective termite infestation observed in the field can be attributed to differential survival in the two timbers. Smythe and Carter (1970), however, demonstrated that differences do exist within tree species of different geographic origin that influence feeding by *R. flavipes*.

The overall high survivorship (range = 73–90%) of *R. hesperus* in *P. menziesii* sawdust is comparable to the high survivorship previously reported for *R. flavipes* (Carter, 1976; Carter and Smythe, 1974; Smythe and Carter, 1970) and *Incisitermes minor* (Hagen) (Kofoid and Bowe, 1934; Rust and Reiersen, 1977) on blocks and sawdusts of this tree species. In contrast, Smythe and Carter (1970) reported poor survival (0–8%) in groups of *Reticulitermes virginicus* Banks on *P. menziesii* heartwood blocks.

Survival in Hardwood Sawdusts. Very few of the workers fed sawdust from either of the two Costa Rican hardwoods *L. seemanii* or *T. ochracea* survived 15 days, and none survived 45 days (Table 1). Mortality in *T. ochracea* sawdust at five days was significantly different from and greater than that in

TABLE 1. PERCENT SURVIVAL OF *Reticulitermes hesperus* WORKERS IN HEARTWOOD SAWDUST OF TWO COSTA RICAN TREE SPECIES

Substrate	Mean survival (% \pm SEM) ^a		
	5 days	15 days	45 days
<i>Lysiloma seemanii</i>	35 \pm 11b	2 \pm 2b	0b
<i>Tabebuia ochracea</i>	5 \pm 4c	0b	0b
α -Cellulose	100 \pm 0a	100 \pm 0a	74 \pm 3a
Starvation Control	99 \pm 1a	97 \pm 3a	0b

^aMean of three groups of 40 *R. hesperus* workers. Means in the same column followed by different letters are significantly different (ANOVA, REGW multiple F test, $\alpha \leq 0.05$).

L. seemanii sawdust, and mortality in both sawdusts differed significantly from that observed in the starvation control. Mortality can thus be attributed to toxicity of the woods rather than feeding deterrence alone, since 15-day survivorship in the sand starvation control was very high ($97 \pm 3\%$) and did not differ significantly from the α -cellulose feeding control ($100 \pm 0\%$).

Survival on Wood Extracts. Survivorship after five days on filter papers treated with each of the six wood extracts (Table 2) was 96–100% and did not differ significantly from survivorship in either the solvent ($99 \pm 1\%$) or starvation ($100 \pm 0\%$) controls. At 15 days, only survivorship on papers treated with extracts of *T. ochracea* ($55 \pm 11\%$) and *P. ponderosa* ($27 \pm 19\%$) heartwoods differed significantly from the solvent control ($98 \pm 2\%$), and only *P. ponderosa* differed from the starvation control ($78 \pm 12\%$). Thus, toxic materials in *P. ponderosa* were extracted in acetone, and toxicants and/or feeding deterrents in *T. ochracea* were extracted in methanol. Compounds extracted from *P. ponderosa* sawdust with a mixture of acetone, hexane, and water have also been found to reduce the survival of *R. flavipes* (Carter, 1976).

The poor survivorship of *R. hesperus* on *T. ochracea* extracts corresponds with the toxicity of the *T. ochracea* sawdust, although the much greater mortality observed in the sawdust assays (Table 1) suggests incomplete extraction of toxicants from both *T. ochracea* and *L. seemanii*. Mortality was virtually complete in all treatments but the solvent control at 45 days (Table 2), and extraction of feeding deterrents from all six woods is supported by the observation that only the control disks had been extensively fed upon. In two-choice feeding assays with the drywood termite *I. minor*, Rust and Reiersen (1977) observed that filter papers treated with methanol extracts of various woods,

TABLE 2. PERCENT SURVIVAL OF *Reticulitermes hesperus* WORKERS ON FILTER PAPERS TREATED WITH WOOD EXTRACTS

Extract	Mean survival (% \pm SEM) ^a		
	5 days	15 days	45 days
<i>Centrolobium</i> sp.	100 \pm 0a	99 \pm 1a	26 \pm 26b
<i>Lysiloma seemanii</i>	96 \pm 2a	95 \pm 1ab	1 \pm 1b
<i>Pinus ponderosa</i>	100 \pm 0a	27 \pm 19c	0b
<i>Pseudotsuga menziesii</i>	97 \pm 0a	60 \pm 7abc	0b
<i>Tabebuia guayacan</i>	100 \pm 0a	72 \pm 11abc	0b
<i>Tabebuia ochracea</i>	97 \pm 1a	55 \pm 11bc	0b
Solvent control	99 \pm 1a	98 \pm 2a	98 \pm 2a
Starvation control	100 \pm 0a	78 \pm 12ab	0b

^aMean of three groups of 40 *R. hesperus* workers. Means in the same column followed by different letters are significantly different (ANOVA, REGW multiple *F* test, $\alpha \leq 0.05$).

including *P. menziesii* and *P. ponderosa*, were not fed upon in favor of untreated or solvent-treated papers.

Group Behavioral Responses. In behavioral assays with groups of ten *R. hesperus* workers (Table 3), only the responses to extracts of *P. menziesii* and *P. ponderosa* differed significantly from the 50% response expected under the null hypothesis of no effect (*t* test, $\alpha \leq 0.05$). In both cases, these were positive responses, suggesting a behavioral preference for filter papers treated with these extracts. Although not differentiated in this assay, such a preference could result either from attraction to the extract-treated papers (orientation to airborne volatiles) or from arrestment after contact (cessation of locomotion), or from a combination of the two behaviors.

Individual Behavioral Responses. The responses of individual workers (Table 3) to extracts of *P. menziesii* and *P. ponderosa* also differed significantly and positively from the no-effect null hypothesis. In these individual assays, the behavioral responses to extracts of *T. guayacan* and *T. ochracea* were also significant, but in a negative direction. Methanol extracts of *T. ochracea* thus appear to contain repellents, in addition to toxic and/or antifeedant materials. The response of *R. hesperus* workers to *P. ponderosa* extract, on the other hand, suggests the presence of positive allelochemical stimuli in addition to toxicants.

Lapachol is considered to be an antitermitic agent in *T. guayacan* (Bultman

TABLE 3. BEHAVIORAL RESPONSES OF GROUPS AND OF INDIVIDUAL *Reticulitermes hesperus* WORKERS TO FILTER PAPERS TREATED WITH WOOD EXTRACTS

Extract	Mean proportional response (\pm SEM) ^a	
	Group assays ^b	Individual assays ^c
<i>Centrolobium</i> sp.	0.48 \pm 0.08	0.46 \pm 0.03
<i>Lysiloma seemanii</i>	0.51 \pm 0.06	0.44 \pm 0.04
<i>Pinus ponderosa</i>	0.73 \pm 0.12 ^d	0.73 \pm 0.04 ^d
<i>Pseudotsuga menziesii</i>	0.78 \pm 0.16 ^d	0.69 \pm 0.03 ^d
<i>Tabebuia guayacan</i>	0.52 \pm 0.14	0.41 \pm 0.04 ^d
<i>Tabebuia ochracea</i>	0.58 \pm 0.06	0.30 \pm 0.04 ^d
Solvent control	0.36 \pm 0.09	0.48 \pm 0.05

^aProportional response indicates the proportion of time spent by the majority of the group or by an individual worker on an extract-treated paper rather than on a solvent-treated control paper in a 20-min, two-choice assay. In the solvent control assays, a "treatment" paper was designated at random.

^bMean of eight groups of 10 *R. hesperus* workers.

^cMean of 50 assays with individual *R. hesperus* workers.

^dMean proportional response is significantly different from the expected mean of 0.50 under the null hypothesis (two-tail *t* test, $\alpha = 0.05$).

and Southwell, 1976; Kukachka, 1970). Becker et al. (1972) found that this compound was repellent to *Microcerotermes crassus* Snyder, and possibly repellent to *Kaloterms flavicollis* (Fabr.), but did not repel the other 12 termite species tested, including three species of *Reticulitermes*. Lapachonone, however, isolated from other *Tabebuia* species, was repellent to 10 termite species, including *R. flavipes*, *Reticulitermes lucifigus* (Rossi), and *Reticulitermes santonensis* Feytaud (Becker et al., 1972).

Several studies have indicated that termites from different colonies may respond differently in bioassays (Carter et al., 1972; Lenz, 1985; Su and LaFage, 1984). Since different *R. hesperus* colonies were used in our behavioral assays with groups and with individual termites, we can make no inferences from these experiments on individual versus group behavior. Problems are apparent, however, with both types of behavioral assays. Rather than averaging individual responses in a single measure of group behavior, or measuring the responses of single termites within the artificial context of an individual assay, an alternative approach of current interest to us is to record the responses of individual termites within the context of a group assay.

CONCLUSIONS

The chemical nature and biological activity of wood extractives have been investigated with respect to termites for over 60 years. Most of this work has utilized tropical hardwoods, with the aim of identifying more resistant building materials (Bultman et al., 1979; Su and Tamashiro, 1986) or isolating toxins or antifeedants that could be applied to susceptible lumber to increase termite resistance (Carter and Beal, 1982).

Only a few researchers (e.g., Carter and Mauldin, 1981; Carter et al., 1983) have investigated behavioral responses to wood extractives in conjunction with feeding assays. Varma et al. (1984) also investigated the attractiveness of extracts of plant materials to *Odontotermes guptai*. It is hoped that the extraction, and subsequent isolation and identification, of behaviorally active compounds from susceptible and resistant woods will contribute to the development of new techniques of behavioral termite control, such as the use of baits (Esenther and Beal, 1979), rather than simply replacing current wood preservatives and insecticides with naturally occurring toxicants.

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BENEFICIAL ARTHROPOD BEHAVIOR MEDIATED BY AIRBORNE SEMIOCHEMICALS

VI. Flight Responses of Female *Microplitis croceipes* (CRESSON), a Braconid Endoparasitoid of *Heliothis* spp., to Varying Olfactory Stimulus Conditions Created with a Turbulent Jet¹

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Abstract—Odor-mediated host-searching by female *Microplitis croceipes*, a braconid endoparasitoid of larval *Heliothis* species, was studied in a flight tunnel. Volatiles emitted by third-instar *H. zea* feeding on cowpea seedling leaves were released and carried with the wind, resulting either in an irregularly shaped plume, or injected with high velocity, resulting in a conically shaped jet plume. Flight maneuvers of *M. croceipes* had a higher stereotype in jet plumes compared to irregularly shaped plumes. Variation in odor concentration in jet plumes due to intermittent feeding of the host larvae did not affect the number of flights. At the levels employed, odor concentration did not alter the rate of upwind progress in jet plumes. Because air velocity inside a jet increases in the upwind direction, *M. croceipes* regulates its ground-speed during its approach to the odor source.

Key Words—Hymenoptera, Braconidae, *Microplitis croceipes*, *Heliothis zea*, Lepidoptera, Noctuidae, anemotaxis, parasitoid, jet plumes, optomotor response, semiochemicals.

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INTRODUCTION

The question of how flying insects orient to volatiles has been studied most extensively in the response of moths to pheromones (Baker, 1986; Cardé, 1984; Kennedy, 1983). However, little is known about behavioral mechanisms that are used by parasitoids during in-flight orientation to host-related odors (Jones, 1986; Waage, 1978). In contrast to sex pheromone communication, in which efficiency in signal emission and response is favored, a larval parasitoid responding to kairomone is confronted with an emitter that benefits by minimizing its detectability.

Microplitis croceipes (Cresson), a parasitoid of *Heliothis* spp., oviposits predominantly in third-instar larvae of *H. zea* (Boddie) (Hopper and King, 1984; Lewis, 1970). These larvae are often concealed inside flower buds and blooms of cotton and prevalent at the lower levels of the canopy (Farrar and Bradley, 1985; Schmidt, 1985), where even a slight breeze will generate abundant turbulence due to the dense vegetation. Powell and King (1984) observed no difference in parasitization rates for host larvae collected from different parts of the plant. This might be due to larval mobility, but it is also possible that *M. croceipes* is equally successful in detecting *H. zea* throughout the plant. The semiochemicals involved in in-flight host-searching of *M. croceipes* are emitted during feeding of *H. zea* larvae on foliage (Drost et al., 1986). Eller et al. (1988) noted that volatile trappings from third instars on cowpeas presented on filter paper were never behaviorally active for more than 10 min.

A question arises whether *M. croceipes* is adapted to shorter detection distances than male moths responding to sex pheromone because turbulence in the habitat disintegrates odor plumes and the host benefits by minimizing its detectability. Of parallel interest is whether flight maneuvers used by *M. croceipes* differ from pheromone-evoked orientation in moths.

Semiochemical-mediated flight of *M. croceipes* was demonstrated in a flight tunnel by Drost et al. (1986). The odor sources used in that study, a cowpea seedling with a feeding third-instar *H. zea*, did not permit systematic manipulation of the odor stimulus. Odors emanating from a plant form an irregularly shaped odor plume, which makes it difficult to determine at which instant the insect is enveloped by the chemical stimulus. The effect of variation in the odor production, due to changes in host feeding, on the flight maneuvers of *M. croceipes* also is unknown. A controlled presentation of stimuli is one of the steps necessary to facilitate the interpretation of flight behavior.

Several systems that allow the use of irregularly shaped odor sources and generate odor plumes with a predictable shape have been developed. These units comprise an enclosed flow system through which clean air is blown over the odor source and discharged into the center of the flight tunnel (Baker and Cardé, 1979; Cardé et al., 1984; Rauscher et al., 1984). In previous studies

these devices have been used to inject the odor-laden air with a velocity equal to or lower than the air velocity in the flight tunnel.

In the present study the odor-laden air is injected with velocities at least 10 times ambient. The difference between ambient and injected flow creates a turbulent jet with a conical shape and sharp but irregularly formed boundaries. A more homogeneous distribution of odor occurs inside a jet plume due to the high level of turbulence, as compared to plumes from an odor source in ambient flow. The term "irregularly shaped," as used in this paper, pertains to odor plumes whose instantaneous dimensions suggest that an insect heading straight upwind towards the odor source will be enveloped intermittently by the chemical stimulus. In contrast, an insect that flies a similar track in a jet plume will be continuously enveloped by the chemical stimulus.

High-velocity injection of odor causes an increase in odor concentration toward the source, similar to other techniques, but is dissimilar in that an insect must increase its flight speed to maintain a fixed ground speed during its approach to the source. Therefore, this odor presentation technique is suited to demonstrate whether insects actively regulate their upwind progress and whether they can maintain this response when approaching the source when odor concentrations are increasing.

The objectives of this paper are to describe in *M. croceipes* the effects on flight maneuvers of: (1) irregularly shaped and jet plumes; (2) intermittent host feeding; and (3) controlled dilution of odors in jet plumes of varying dimensions.

METHODS AND MATERIALS

Insects. *H. zea* larvae were reared on artificial diet using the method of Burton (1969). A colony of *M. croceipes* was obtained in March 1984 from the Southern Field Crop Insect Management Laboratory, Stoneville, Mississippi. This colony had been in culture at Stoneville since September 1982 and reared on *H. zea* (Lewis and Burton, 1970) in the Insect Biology and Population Management Research Laboratory, Tifton, Georgia. Parasitoids of both sexes were kept in Plexiglas cages (30 × 30 × 17 cm) at 28°C, 50–70% relative humidity, a 8:16 dark–light photocycle, and provided daily with fresh water and honey. Mating occurred as soon as both sexes were present (Bryan et al., 1969). The age of the females used in our experiments varied between 3 and 7 days. All experiments were done on at least three different days, and we used parasitoids from different collections each day.

Odor Sources. Greenhouse-reared cowpea seedlings (*Vigna unguiculata* L.), 5–10 cm in height, were used in all experiments. Either third- or fifth-instar *H. zea* feeding on cowpea seedling leaves were used as odor sources.

Both instars are attacked in the field (Hopper and King, 1984) and host viable progeny. To ensure feeding activity during the experiments, we starved the *H. zea* larvae during the 20 hr preceding the test: the third and fifth instars were provided with only one third or one half of a cowpea seedling leaf, respectively.

The average feeding rate for starved third- and fifth-instar *H. zea* larvae was obtained by timing the cumulative feeding duration and associated weight loss of cowpea seedling leaves. We observed individuals of each instar for 2 min. Third-instar *H. zea* consumed 4.1 ± 0.6 mg/min (mean \pm SE, $N = 14$) and fifth instars 23.5 ± 10.8 mg/min (mean \pm SE, $N = 24$) cowpea foliage. An odor source with 16 third instars will contain feeding activity approximately equivalent to three fifth instars, but presumably with lower variation over time.

To study the effect of intermittent feeding, we used individual fifth instars as an odor source because feeding was more visible on video recordings. To study in-flight navigation with a constant odor source, we used a group of 16 third instars.

The effect of daily variation in average feeding rates on the percentage of sustained flights was tested using a maximum likelihood estimate for a standard response function as provided by SAS (1982).

Flight Tunnel. An open-circuit, closed-throat flight tunnel with a $50 \times 50 \times 100$ -cm working section, described previously by Drost et al. (1986), was modified as follows: a 10×10 -cm grid of black lines (4 mm width) on white posterboard was placed underneath the Plexiglas floor; also the motor that drives the fan was held with rubber blocks to reduce vibrations of the flight tunnel floor. The air velocity was 50 ± 2 cm/sec (mean \pm SE, $N = 25$), the temperature was 26°C and relative humidity was 40–70%. Overhead lighting was provided by two fluorescent bulbs. The light intensity varied between 2260 and 3010 lux, depending on the position on the flight tunnel floor.

Contamination of the air circulating in the flight tunnel was minimized by releasing semiochemicals only when a parasitoid was to be tested. Between tests the air velocity in the flight tunnel was set at maximum speed to enhance ventilation of the test section. During experiments, the air of the room that housed the flight tunnel was continuously exhausted outside the building. The flight tunnel was cleaned using the procedures described by Drost et al. (1986).

Odor Supply System. An enclosed flow system (Figure 1) holds an odor source and injects emitted semiochemicals with the wind in the flight tunnel. Air, pressurized with a membrane pump (model 7530-40, Cole Parmer), was filtered through activated charcoal, kept at ambient temperature, and humidified with distilled water. Air in the supply system was heated by the membrane pump, and to prevent condensation of warm humidified air in the nozzles, the air was cooled to ambient levels. Connections up to the odor source were made of Tygon (B-44-3) tubing. Beyond the odor source we used 3-mm ID glass tubing. The main valve of the system, a three-way stopcock, allowed simulta-

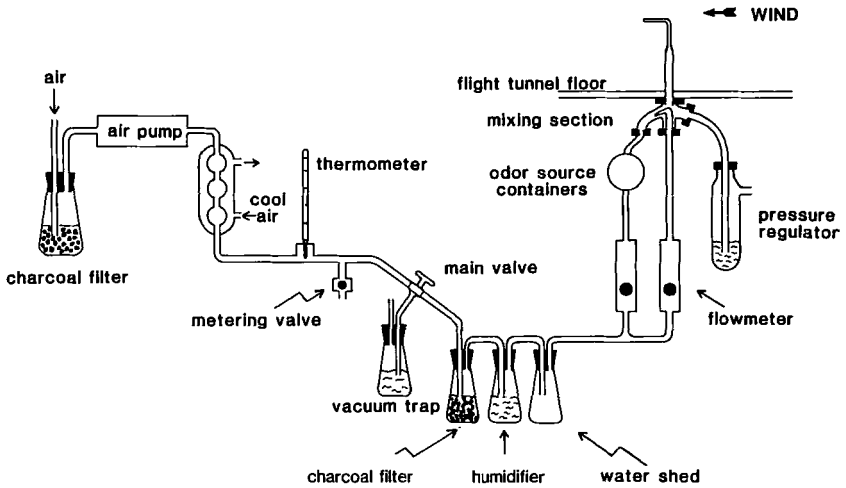


FIG. 1. Schematic of the odor supply system.

neous disconnection of the air supply and depressurization of the flow system. Because the air was pulled through the flight tunnel causing an inside pressure lower than ambient, the odor supply system was depressurized through a vacuum trap to prevent leakage of odor when the main valve was closed. Flow rates were monitored and adjusted separately for the branch containing the odor source and the diluting branch with two flowmeters (model 7530-40, Cole Parmer). All experiments were done with a set flow rate of 244 ml/min in the branch containing the odor source. The odor concentration in the jet plume could be lowered by adding clean air to the odor-laden air in the mixing section. During dilution a pressure relief valve made of an adjustable air bubbler (model 7506-06, Ace Glass) kept the pressure in the system at a preset level. This method of dilution allowed us to manipulate shape and odor concentration of the jet plume independently.

The odor collection container designed to monitor larval behavior was constructed as follows. Three No. 8 rubber stoppers were connected in series with 3-mm ID glass tubing and could be covered with plastic Petri dishes (model 1008, Falcon). Each Petri dish could hold a cowpea seedling leaf and a fifth-instar *H. zea*. The space between the Petri dish and the stopper restricted the larva in one plane and thereby minimized the possibility of concealment during feeding. The total capacity of this container was 13 ± 0.5 ml.

The odor collection section used for navigation studies consisted of two units. Each unit held four 1-dram vials (6 cm height, 1.5 cm diam.) in two parallel groups of two vials connected in series. To reduce cannibalism and fighting, we used two larvae per unit of the odor collection container. In pilot

tests we found that the frequency of landings decreased on odor sources that contained fighting or cannibalizing larvae. The total capacity of the collection section with a set of eight units, containing eight cowpea seedling leaves and 16 third-instar *H. zea*, was 82 ± 0.5 ml.

Connected to the mixing section was a nozzle made of a Pasteur pipet, which injected odors in the main wind direction of the flight tunnel, 10 cm above the floor. To provide a landing site, a cardboard target was attached to the nozzle and positioned inside the plume but outside the high-speed flow region (Figure 2). *M. croceipes* lands on any object that is provided with host-related volatiles (Drost et al., 1986).

Odor Presentation. The shape and average velocity distribution in a turbulent jet are determined by the difference in velocity of injected and ambient air and the diameter of the nozzle. We obtained a narrow, low-velocity jet with a low-pressure nozzle and a wide, high-velocity jet with a high-pressure nozzle. A high-pressure nozzle was made by heating and simultaneously pulling the tip of a Pasteur pipet (model P5200-2, diSPo pipets, Scientific Products). High-pressure nozzles had an over pressure of 6 ± 0.5 cm water above atmospheric pressure, at a flow rate of 244 ml/min. The tip of a Pasteur pipet was left unaltered for a low-pressure nozzle. The average air velocities in the turbulent

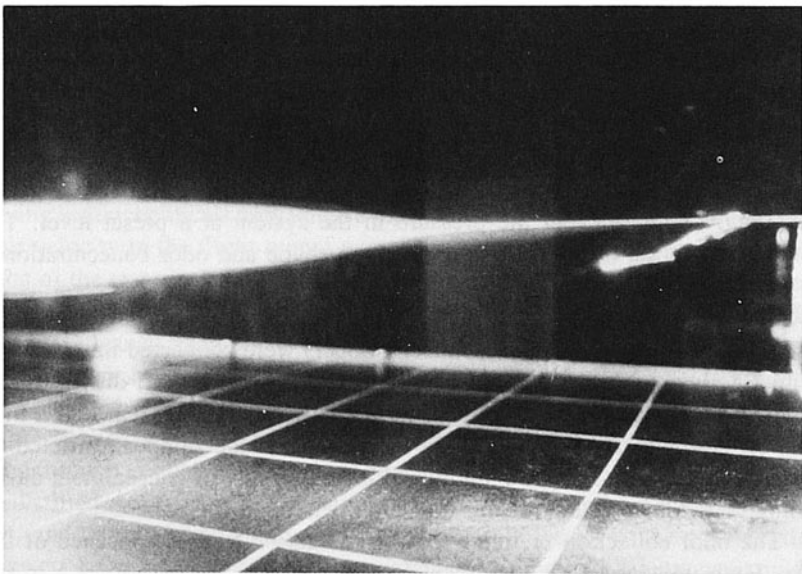


FIG. 2. Wide jet plume visualized with titanium chloride smoke and position of landing site attached to the nozzle.

jets were measured on the center line of the jet at 10 cm intervals with a hotwire anemometer (model AB-27, Hastings Raydist, Hampton, Virginia).

The time-averaged dimension of a cross section of a jet plume was obtained using a method similar to the ones described by Miller and Roelofs (1978) and by Sanders (1985). A frame covered with cheesecloth, with dimensions that fitted exactly inside the tunnel, was positioned at appropriate distances from the nozzle. The air-pull of the fan of the flight tunnel was adjusted so that drag losses caused by the screen were corrected to an air velocity of 50 cm/sec upwind of the screen. Cheesecloth impregnated with crystal violet was placed downwind from the nozzles at the upwind side of the screen. Acid "smoke," resulting from titanium tetrachloride reacting with humidified air, was injected and the acid component stained the purple cheesecloth yellow according to the size of the jet plume. After a 3-min exposure to the acid smoke, a print of the cross section of a jet plume was immediately photographed. The dimensions of the turbulent jets were sampled at the same 10-cm intervals used in the air velocity measurements.

Event Recorder. An event recorder program in BASIC for a Tandy model 100 computer allowed the sequential recording of behavioral components and their duration in seconds, the "complete" record (Colgan and Smith, 1978) and the experimental conditions. To describe each experiment, we used the variables of date, time of day, parasitoid age, ambient temperature, type of odor source, type of flight response, individual number, and trial number. The following behavioral components applicable in a flight tunnel, modified from Drost et al. (1986), were recorded: (1) preflight behavior—all behavior between first contact with the odor plume and take-off; (2) initial zigzagging—lateral excursions, mainly in the horizontal plane and perpendicular to the wind direction soon after take-off; (3) straight flight—straight upwind flights; (4) recurrent zigzagging—lateral excursions after straight flight; (5) downwind loop—a downwind looping maneuver; (6) hovering—stationary flight at approximately 10 cm downwind from the target; and (7) standing still on the target—standing still on the target.

After analysis of our raw data, we found that during sustained flights initial zigzagging was always followed by straight flight. Therefore, in behavioral descriptions the term *initial flight stage* is used instead of the sequence initial zigzagging; straight flight.

Each observation was terminated after a landing occurred on a wall of the flight tunnel or on the target and given a response category according to the types of behavioral components performed.

The following response categories were used: (1) sustained flight—nonstop flights resulting in standing still on the target; (2) temporary-oriented flight—flights that included at least initial zigzagging and straight flight but were subsequently terminated; (3) non-target-oriented flight—flights terminated imme-

diately after or before initial zigzagging; and (4) no take-off—preflight behavior lasting longer than 2 min. Behavioral sequences of females performing the response category sustained flight to the same type of odor source were used to construct contingency tables of transition frequencies between behavioral components.

Statistical Analysis of Behavioral Sequences. Conditional probabilities of transitions between behavioral components were calculated to depict the main behavioral pathway. We tested whether transitions occurred at a frequency significantly different from chance expectation assuming independence between behavioral components. For behavioral components preceded or followed by more than two other types of components, we calculated standardized residuals based on estimated expected frequencies obtained by iterative proportional fitting (Bishop et al., 1975; Goodman, 1968), as provided by SAS (1982). Because certain behavioral components cannot follow each other, as a consequence of their definition (see Figure 2 in Drost et al., 1986), and not themselves as a result of our sampling procedure, the contingency tables were incomplete due to “logical zeros” (Bishop et al., 1975). In this case the iterative procedure provides row and column sums of estimated expected frequencies that converge to the corresponding sums of the observed frequencies, a requirement for the use of chi-square. Because initial flight stage was only preceded by preflight behavior, following its definition, this transition was not eligible for the chosen analysis and omitting this transition resulted in a similar situation for preflight behavior. Therefore behavioral components following preflight behavior were analyzed using a binomial test.

When the absolute value of the standardized residual, $(\text{observed} - \text{expected})/[\text{SQRT}(\text{expected})]$, of a transition frequency exceeded 3, then that transition occurred at a frequency significantly different from chance expectation assuming independence between behavioral components (Colgan and Smith, 1978). Because we performed multiple comparisons, the 1.96 standard can serve only explorative means, and standardized residuals with values greater than 3 are a more reasonable guide for significance. We used contingency tables with more than five times the possible transitions (19) observed. This amount (95) is a recommended minimum required for the use of the standardized residuals method (Colgan and Smith, 1978).

Statistical Analysis of Durations of Behavioral Components. The precision of the stopwatch of the event recorder is 1 sec. We chose to use only those durations whose range was 30 sec or more or had a median of at least 5 sec.

To test whether durations of behavioral components were independent from following components, durations were plotted semilogarithmically in a log-survivor plot. If, for each given behavioral component, the probability to occur is constant through time (a Poisson process), then the duration of components will have an exponential distribution and the log-survivor curve will be a straight

line. The Kolmogorov goodness-of-fit test was used to test whether log-survivor curves differed significantly from a straight line (Colgan and Smith, 1978; Conover, 1971).

The mean duration of each occurring behavioral component was calculated for each sustained flight. The effect of treatments on the mean duration of behavioral components was tested using the Wilcoxon test in case of a two-level comparison or the Kruskal-Wallis test when more levels were to be tested.

Video Recording. A video camera (model TC 2055/UBC, RCA), connected to a video recorder (model NV-8950, Panasonic), with a 8 mm f1.4 lens (model TC 1810C, RCA) was mounted in front of the larval container or on top of the flight tunnel. The video recorded an area of 40×90 cm of the flight tunnel floor. A time generator (model WJ-810, Panasonic) was used to superimpose hundredths of a second onto the video signal. Flight tracks of sustained flights, performed by first-time flyers, were traced on acrylic sheets. Subsequently these tracings were measured with a digitizer (model HP-9874A, Hewlett-Packard) interfaced to a computer (model HP-9915A, Hewlett-Packard) using software written in BASIC.

Flight track measurements were made relative to the 10×10 -cm grid on the floor of the flight tunnel. Because we wanted to determine the relationship between position in the plume and flight behavior, measurements were allocated to squares enclosed by the grid, numbered one (10 cm downwind from the odor source) through seven (near the release point, below the jet plume). The time and position of an insect's exit from a square were used to allocate tracks to the squares. Flight tracks that included downwind loops extending beyond one square were not used in the analysis. Distortion of the grid in the area of flight activity was of the same order of magnitude as the body length of the insect and was therefore ignored.

The average ground speed, the distance traveled, and the maximal flight deviation from the center line of the odor plume were measured for each square (Figure 3). Average ground speed in a square was calculated as the quotient of the track length and time elapsed to travel that distance. The maximal flight deviation from the center line of the plume gives the envelope within which the parasitoid flew. Distance traveled increases the more the insect deviates from a straight flight path and is thus related to the frequency and amplitude of zig-zagging. When the insect flies in a straight line from the release point to the target, distance traveled is the minimum of 70 cm.

We found the variation of log $(x + 1)$ -transformed data for ground speed and maximal flight deviation to be normally distributed using the Shapiro-Wilks (1965) statistic as provided by SAS (1982). This was not the case for distance traveled. Consequently, we used an ANOVA for ground speed and maximal flight deviation after testing for homoscedasticity and the Kruskal-Wallis test for distance traveled to test for homogeneity among days for each square of

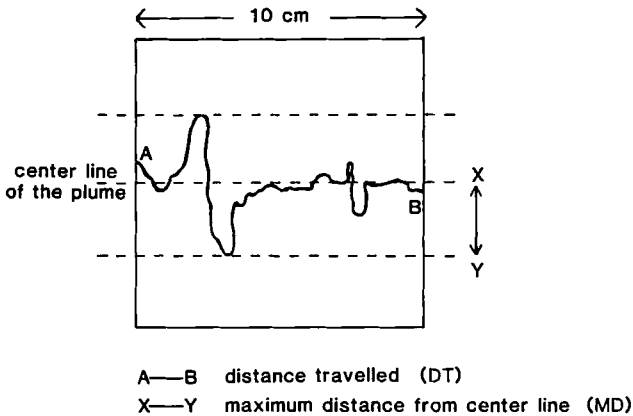


FIG. 3. Schematic of the flight track measurements made in each square.

each treatment. Further analysis was performed on data for which homogeneity among days was obtained. Significance of differences among treatments, among squares within a treatment, and among treatments for each square for ground speed and maximal flight deviation was tested using an ANOVA and the Duncan's multiple-range test on $\log(x + 1)$ -transformed data, whereas the Friedman test was used in the case of distance traveled.

General Experimental Procedure. All experiments took place between the 4th and 8th hour of the photophase. Odor sources were prepared as follows. Shortly before adding the larvae, cowpea seedling leaves were picked and weighed. The period that larvae and cowpea foliage were together in the odor collection container was timed in minutes. Immediately after the completion of the experiments, the leaves were weighed again and the weight loss of the leaves was calculated. The average feeding rate was used to compare odor production during different experiments.

Prior to testing, the odor supply system was left open for 30 min to allow the equilibration between release and adsorption of semiochemicals on the glass walls of the supply system. Pasteur pipets were rinsed with acetone after use.

About 30 min prior to the experiments, a cage with untested parasitoids was transferred from the rearing room to the flight tunnel room. Prior to release in the flight tunnel, female parasitoids were exposed for 2 min to a third-instar *H. zea* and frass as described by Drost et al. (1986). These third-instar larvae had fed on cowpea seedling leaves overnight. Females were discarded if they were injured by the host larva during their stay in the exposure cup or performed prolonged supination of the wings. The latter is a response to being doused by fluids of a larva when it regurgitates in response to being stung. A 1-dram vial

(8 × 1 cm), containing a parasitoid with preflight exposure, was placed with its opening 80 cm downwind from the odor source. As soon as the parasitoid reached the opening of the vial, the video recorder was started, the main valve of the odor supply system was opened, and the recording of the behavior with the event recorder commenced. When a parasitoid landed, recording was stopped, the main valve of the odor supply system was closed, and the parasitoid was caught with the release vial and returned to the initial release point for a next trial.

RESULTS

Experiment 1: Flight in Irregularly Shaped Odor Plumes. Thirty female parasitoids were released individually in odor plumes generated from third- or fifth-instar larvae on a cowpea seedling placed at the center of the flight tunnel at the upwind side. Air velocity was 50 cm/sec. Each female was given four trials, and flight behavior was recorded with the event recorder.

We observed no significant (binomial test, $P < 0.05$) differences in percentages of sustained flights in irregularly shaped plumes emitted by either third (69%, $N = 120$) or fifth (63%, $N = 120$) instars feeding on cowpea seedling leaves.

Flight behavior during sustained flights remained similar among trials in terms of frequencies of transitions between behavioral components and were not significantly different for flights toward both third and fifth instars ($\chi^2_{54} = 40.8$ and 31.5 , respectively). The duration of the initial flight stage of sustained flights to third instars declined significantly ($P < 0.0004$) over the first, second, and third trials, which matches the observations of Drost et al. (1986). The durations of all behavioral components during sustained flights to fifth instars did not differ among trials.

From the conditional probabilities shown in Figure 4A, it follows that sustained flights in irregularly shaped plumes, permeated with semiochemicals from third-instar larvae feeding on a cowpea seedling, were most frequently performed in the following behavioral sequence: preflight behavior, initial flight stage, recurrent zigzagging, straight flight, standing still on the target. Behavioral components that did not occur independently are summarized in Table 1. Transitions from downwind loop to recurrent zigzagging and from hovering to standing still on the target had standardized residuals larger than 3. Transitions between preflight behavior and initial flight stage occurred more often than can be expected by chance (binomial test, $P < 0.05$).

Experiment 2: Effect of Intermittent Host Feeding on Flight. Flight tunnel responses of female *M. croceipes* and the behavior of the larvae used for the

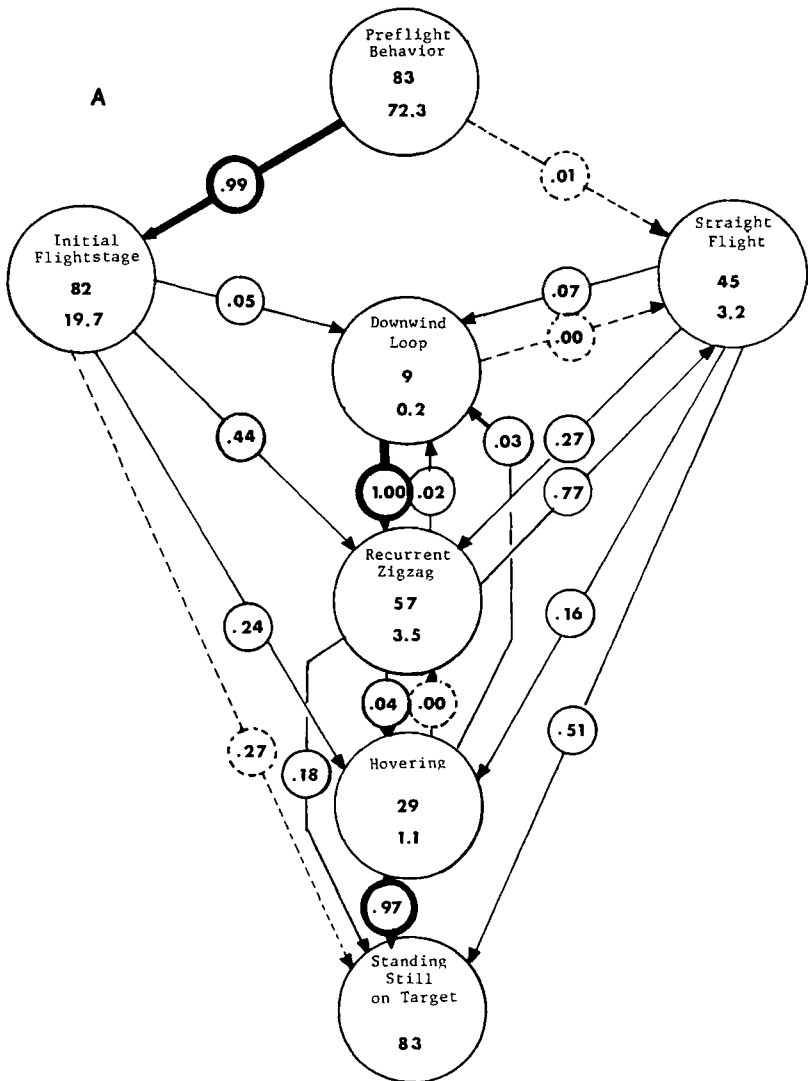


FIG. 4. Kinematic graphs of transitions between behavioral components performed during sustained flights toward semiochemicals emitted by third-instar *H. zea* feeding on cowpea seedling leaves when presented an irregularly shaped plume (A, $N = 83$) and a jet plume (B, $N = 83$). Information provided inside large circles is: behavioral component, number of tokens, and percent of total time spent to that behavioral component. Numbers in small circles associated with arrows are conditional probabilities. Boldface arrows and circles refer to transitions that occur more often than random, whereas dotted arrows refer to transitions that occur less often than random.

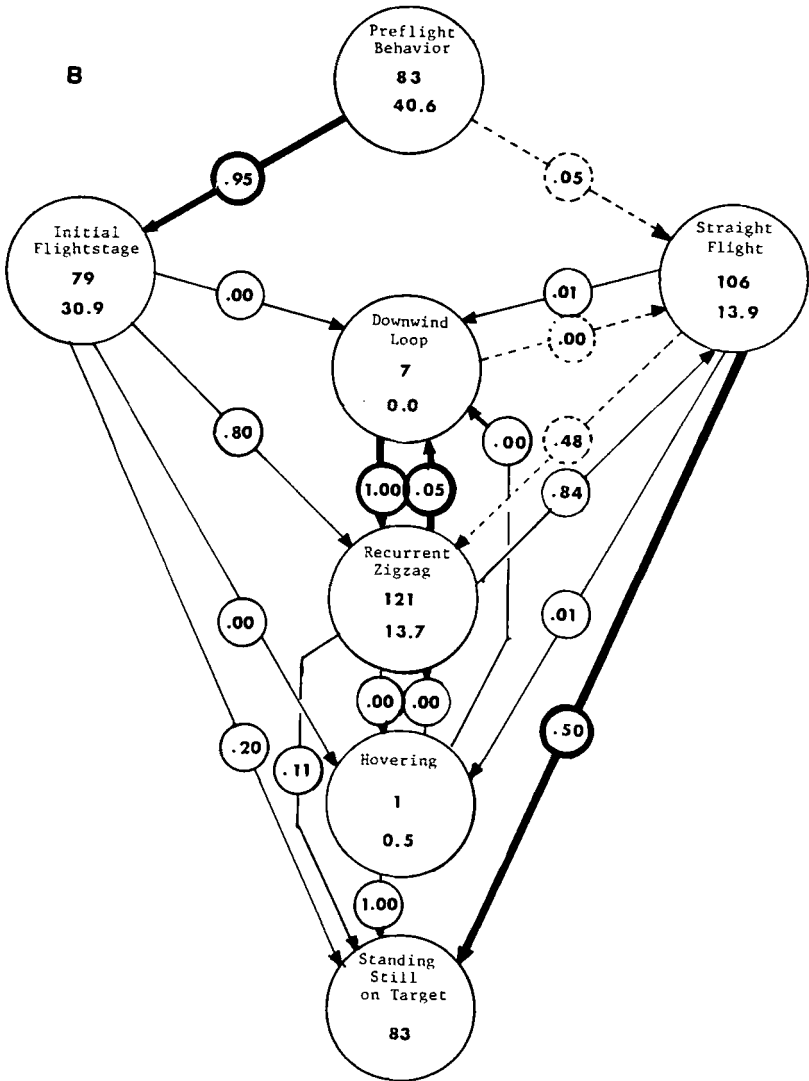


Fig. 4. Continued.

odor source were recorded simultaneously. The onset and duration of feeding movements by the fifth-instar larvae were determined by frame-by-frame analysis of the video recordings.

Of the 80 tested parasitoids, three were released when two larvae were feeding at the same moment, one when three larvae were feeding simulta-

TABLE 1. FIRST-ORDER TRANSITIONS BETWEEN BEHAVIORAL COMPONENTS DURING SUSTAINED FLIGHTS OF *Microplitis croceipes* TOWARD *Heliothis zea* FEEDING ON COWPEA SEEDLING LEAVES PRESENTED IN AN IRREGULAR SHAPED PLUME

Preceding behavior	Statistic ^a	Following behavior					Total
		III	IV	V	VI	VII ^b	
II Initial flight stage	obs		36	4	20	22	82
	exp		27.79	3.83	15.10	35.28	82.00
	StdR		1.56	0.09	1.26	-2.24	
III Straight flight	obs		12	3	7	23	45
	exp		15.25	2.09	8.29	19.36	44.99
	StdR		-0.83	0.62	-0.45	0.83	
IV Recurrent zigzagging	obs	44		1	2	10	57
	exp	36.83		1.42	5.62	13.13	57.00
	StdR	1.18		-0.35	-1.53	-0.86	
V Downwind loop	obs	0	9				9
	exp	7.03	1.97				9.00
	StdR	-2.65	5.00				
VI Hovering	obs		0	1		28	29
	exp		12.05	1.66		15.29	29.00
	StdR		-3.47	-0.51		3.25	
Total		44	57	9	29	83	222

^aobs = observed frequency; exp = expected frequency; StdR = standardized residual.

^bVII: Standing still on the target.

neously, and the remaining 76 when no feeding or feeding by a single larva was observed. The median duration of feeding bouts for an individual larva was 2 min (range 1-19 min) and the median duration between feeding bouts was 5 min (range 1-15 min). Between feeding, larvae were defecating, sitting motionless, walking, and making jabbing movements of the head.

Apparently in our experimental set-up there was no decay of attractancy between feeding. Sustained flights were performed by 49% ($N = 76$) of the wasps evaluated. There were no significant differences in the percentage of sustained flights during feeding bouts (50%, $N = 29$) compared to periods between feeding bouts (48%, $N = 47$); between percentages of sustained flights during feeding bouts longer than 2 min (upper 50% percentile) and shorter than 2 min; and between the upper and lower 50% percentile of periods between feeding bouts (median 5 min).

Flight behavior was not different for periods with and without host feeding, because no significant difference was found for transition frequencies between behavioral components ($\chi^2_{18} = 9.829$).

The log-survivor curves of durations of preflight behavior and the cumulative duration of all other behavior during sustained flights did not significantly differ from a straight line, which means that the durations of these behavioral components are independent from preceding or following components (Hooff, 1982). More parasitoids performed shorter preflight behavior when responding during host feeding bouts compared to those that responded between feeding bouts, as expressed in the different slopes of the log-survivor curves (Figure 5). However, we found no significant differences for the average durations of pre-flight or flight-behavior components for sustained flights either during or between feeding bouts.

Experiment 3: Effect on Flight of Varying Olfactory Conditions in Jet Plumes. Six types of odor plumes were presented in a randomized order, and five parasitoids were released twice into each plume. The experiment was replicated six times. Odor sources used for jet plumes were: humidified air as a control; semiochemicals emitted by 16 third-instar *H. zea* feeding on cowpea seedling leaves (two larvae per leaf); and the same amount of odors diluted by adding an equal amount of clean air, as described earlier. Odors were presented either by a "wide" or a "narrow" conical-shaped turbulent jet. When an odor plume had a lower odor concentration than one previously presented, the branch of the odor supply system containing the odor source was closed and clean air provided by the diluting branch was ducted through the nozzle for 15 min prior to testing.

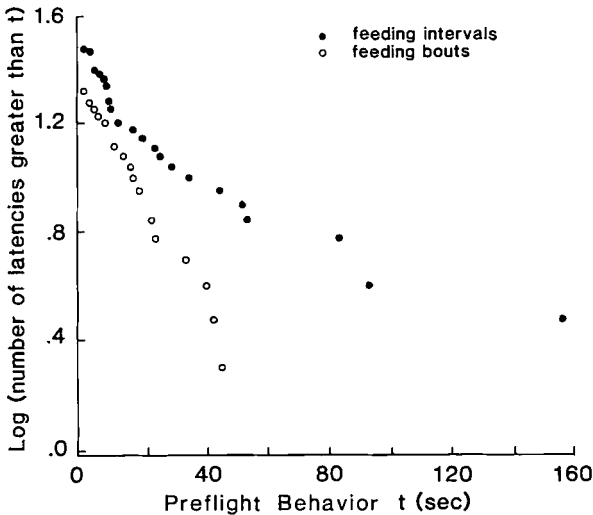


FIG. 5. Log-survivor curve of preflight behavior prior to sustained flights by *M. croceipes* during feeding bouts and feeding intervals of the "odor-source larvae."

The daily variation in feeding activity for a group of 16 third instars did not significantly affect the percentage of sustained flights to each treatment. The percentage of sustained flights, however, did vary for the six types of jet plumes (Figure 6A). No sustained flights were observed with either control plume (low- or high-pressure nozzles injecting humidified air). A significantly lower percentage of sustained flights was found for a narrow jet plume with a half odor load when compared to a wide jet plume with a full odor load. Of all unsuccessful responses the category "nonoriented" was the most frequent, whereas temporary-oriented flights seldom occurred (Figure 6B).

Behavioral components performed during sustained flights in the four odor-containing types of jets were compared. Although jets with half odor loads evoked significant lower percentages of sustained flights, when those flights occurred, no significant difference was found in frequencies of transitions between behavioral components as compared to those performed in jets with full odor loads ($\chi^2_{54} = 31.85$).

From the conditional probabilities shown in Figure 4B, it follows that sustained flights in all four types of jet plumes, permeated with semiochemicals from third-instar larvae feeding on cowpea seedling leaves, were performed with less variation in the behavioral sequences than in irregularly shaped plumes of the same odor source. More behavioral components during sustained flights in jet plumes did not occur independently as compared to sustained flights in irregularly shaped plumes. Table 2 shows that the transitions between downwind loop and recurrent zigzagging and straight flight into standing still on the

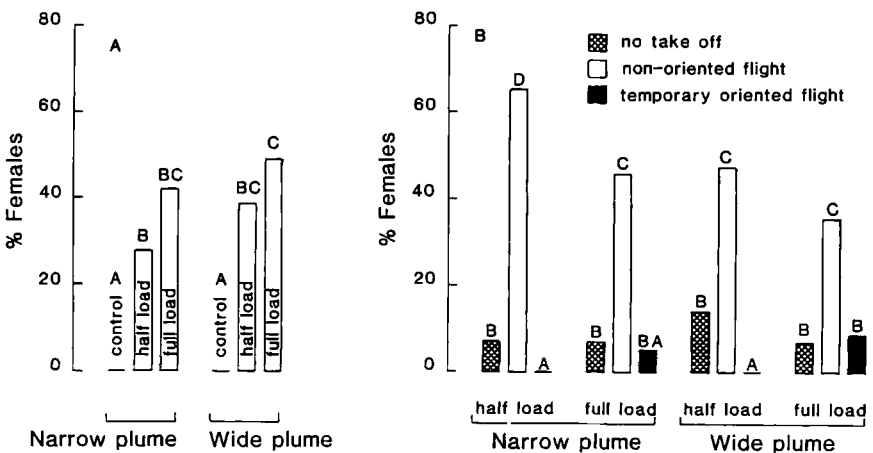


FIG. 6. Percentages of sustained flights (A) and nonsustained flights (B) in two sizes jet plumes with full and half odor loads. Bars associated with different letters are significantly different with chi square ($P < 0.05$).

TABLE 2. FIRST-ORDER TRANSITIONS BETWEEN BEHAVIORAL COMPONENTS DURING SUSTAINED FLIGHTS OF *Microplitis croceipes* TOWARD *Heliothis zea* FEEDING ON COWPEA SEEDLING LEAVES PRESENTED IN JET PLUME

Preceding behavior	Statistic ^a	Following behavior					Total
		III	IV	V	VI	VII ^b	
II Initial flight stage	obs		63	0	0	16	79
	exp		50.53	2.19	0.31	25.96	78.99
	StdR		1.75	-1.48	-0.56	-1.96	
III Straight flight	obs		51	1	1	53	106
	exp		67.81	2.94	0.42	34.84	106.01
	StdR		2.04	-1.13	0.89	3.04	
IV Recurrent zigzagging	obs	102		6	0	13	121
	exp	96.99		1.85	0.26	21.90	121.00
	StdR	0.51		3.06	-0.51	-1.90	
V Downwind loop	obs	0	7				7
	exp	4.86	2.14				7.00
	StdR	-2.21	3.33				
VI Hovering	obs		0	0		1	1
	exp		0.64	0.03		0.33	1.00
	StdR		-0.80	-0.17		1.16	
Total		102	121	7	1	83	314

^aobs = observed frequency; exp = expected frequency; StdR = standardized residual.

^bVII: Standing still on the target.

target had standardized residuals larger than 3. The transitions from preflight behavior into initial flight stage occurred more often than can be expected by chance (binomial test, $P < 0.05$).

The log-survivor plot of durations of behavioral components performed in the four odor jet plumes did not differ from a straight line, so apparently there was no dependency of durations between behavioral components. The average durations of behavioral components were compared and found not to differ significantly among the four treatments.

A Comparison of Flight in Irregularly Shaped Plumes and Conically Shaped Jet Plumes. We obtained percentages of sustained flights in irregularly shaped plumes generated with third instars (69%, $N = 120$) or fifth instars (63%, $N = 120$) on cowpea seedlings similar to those of Drost et al. (1986) (58%, $N = 24$). The percentage of sustained flights was lower when odors from similar sources (third: 40%, $N = 83$; fifth: 49%, $N = 76$) were released in jet plumes. *M. croceipes* is confronted in the field with odor plumes typical for point sources, which resemble irregularly shaped plumes. However, jet plumes

provide a continuous odor stimulus, which may have caused a lower percentage of sustained flights.

Contingency tables of behavioral components performed during sustained flights in irregularly shaped plumes and jet plumes (Figure 4A and B), both permeated with semiochemicals from feeding third-instar larvae on cowpea seedling leaves, differed significantly ($\chi^2_{18} = 123.7$). During sustained flights in jet plumes the initial flight stage was more often followed by the behavioral sequence of recurrent zigzagging, straight flight than during sustained flights in irregularly shaped odor plumes. In irregularly shaped odor plumes the initial flight stage was more often followed by hovering, whereas straight flight was more often followed by downwind loops. The duration of preflight behavior lasted significantly longer for parasitoids responding with sustained flights in irregularly shaped plumes as compared to jet plumes ($P < 0.0001$).

Flight Track Analysis of Video Recordings. To compare responses of the parasitoids in different portions of the jets, it is necessary to take into account the different levels of olfactory stimuli. However, because no prediction of actual odor emission rates of the odor sources used are available, we calculated relative expected odor fluxes. We used the position in the jet 10 cm downwind from the nozzle with the highest expected odor flux as a reference, i.e., vertically above the first square inside a narrow jet plume permeated with a full odor load. Local odor flux in a jet plume varies with the inverse of the local cross-sectional area of the jet times the local air velocity. To calculate local cross-sectional area, we used the time-averaged dimensions of the jet plumes obtained with the titanium tetrachloride staining method (Figure 7). The average air velocity in a jet plume varies from ambient at the boundaries to a maximum at the center line of the jet. The center line odor flux is encountered by an insect when sitting on a release vial at the center line of the jet. For the release point, in the four jet plumes, these odor fluxes were, respectively, 1.5, 0.75, 0.50, and 0.25% of the reference odor flux for a narrow plume full load, a narrow plume half load, a wide plume full load, and a wide plume half load. When an insect starts flying, odor fluxes perceived instantaneously are difficult to estimate, because in a jet plume the air velocity drops from the local maximum at the center line to ambient at the boundaries. However, an insect flying upwind with a fixed average flight speed relative to the ground will perceive, on average, increasing odor fluxes of 32- to 67-fold close to the center line of the jet plume, as depicted in Figure 8. In the case of boundary following, the average increase of the perceived odor flux will be at least 20-fold, because turbulent mixing causes a homogeneous distribution of odors inside the jet plume and air velocity will average equal to ambient.

The plume width and maximal flight deviation from the center line of the plume is plotted against square number in Figure 9A. No significant differences

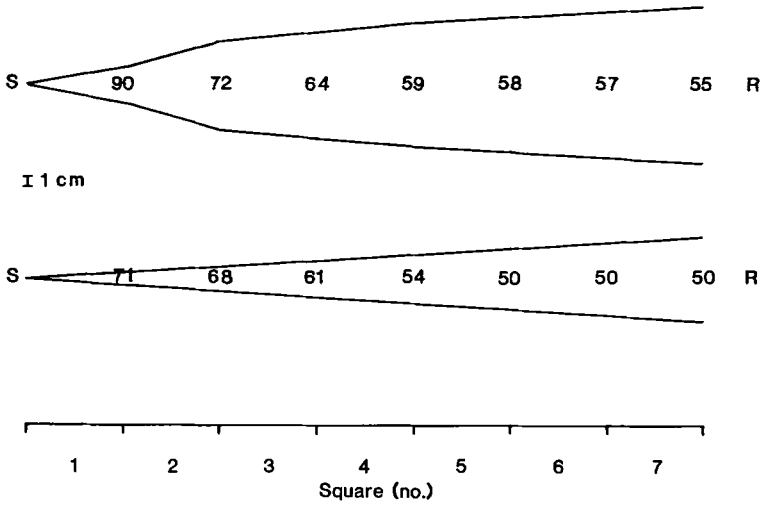


FIG. 7. Dimensions (cm) of wide and narrow jet plumes. Numbers at center line of each jet plume refer to measured air velocities (cm/sec), S = source, R = release point.

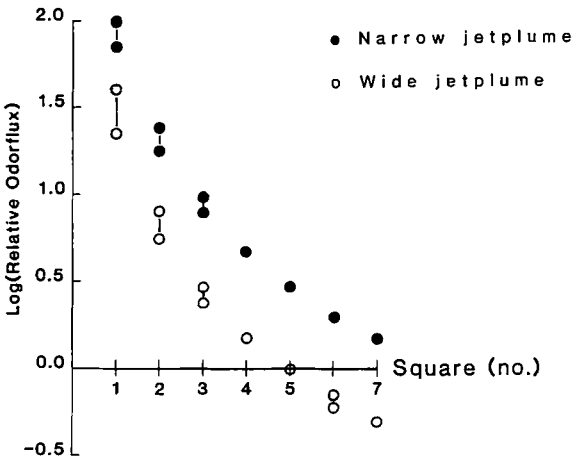
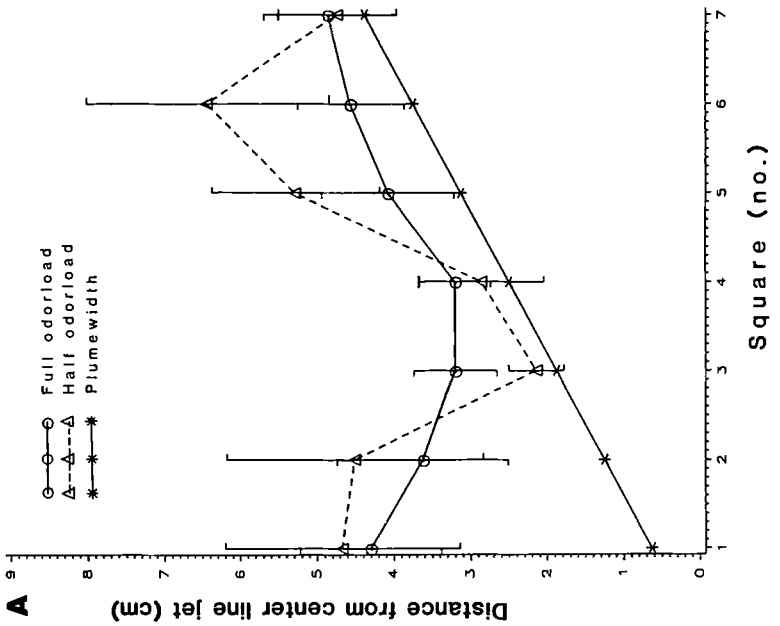
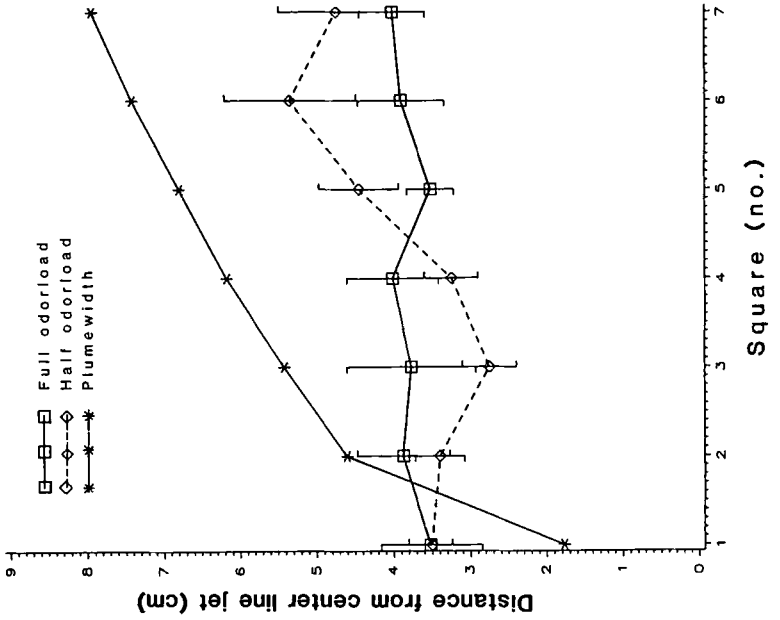


FIG. 8. Estimates of olfactory stimulus conditions occurring in wide and narrow jet plumes with full (upper symbols) and half (lower symbols) odor loads expressed in relative odor fluxes above each square.



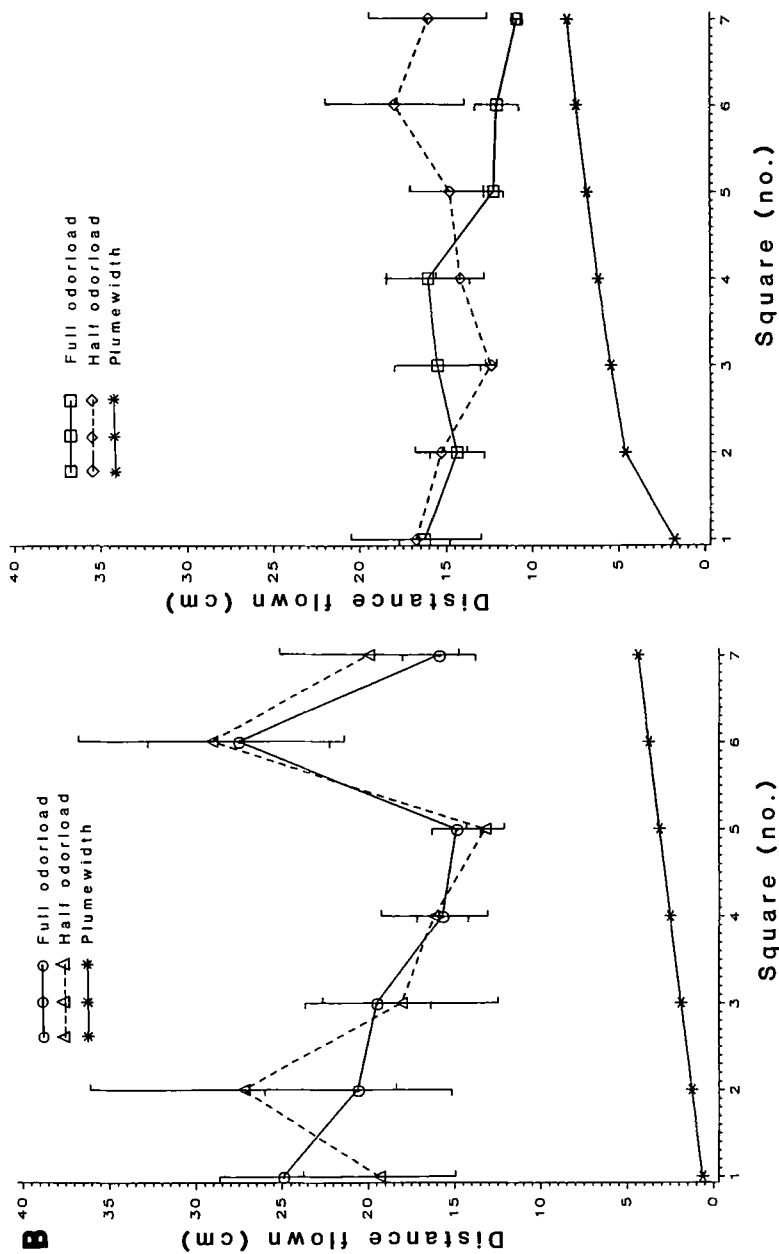


FIG. 9. Measurements from videotaped flight tracks in a narrow jet plume (left graph) and wide plume (right graph). (A) Maximal flight deviation from center line plume and plume width. (B) Distance traveled and plume width. (C) Average ground speed and air velocity at center line plume.

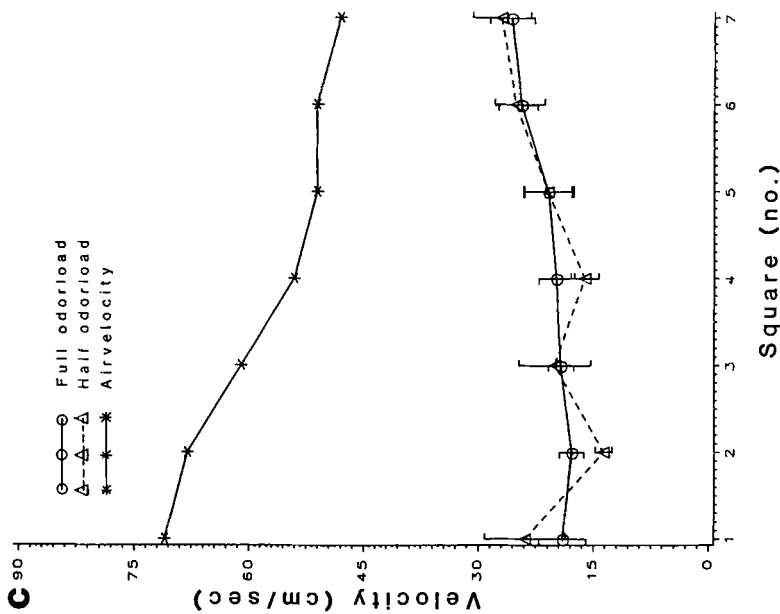
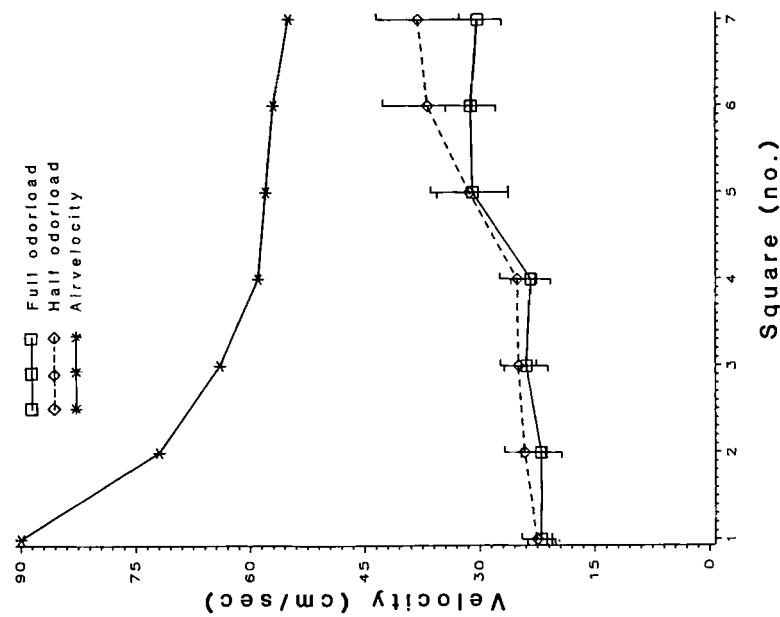


Fig. 9. Continued.

for maximal flight deviation between treatments were found. Unfortunately, part of the flight behavior in wide plumes was not recorded. In contrast to initial zigzagging performed in narrow plumes, *M. croceipes* tended to drift quickly downwind from the release point during initial zigzagging in wide plumes and out of the viewing area of the video camera. Apparently, straight flight and recurrent zigzagging were performed similarly in narrow and wide plumes, whereas initial zigzagging in a narrow plume did not differ in its amplitude from recurrent zigzagging.

From these findings it remains questionable whether *M. croceipes* uses information on the position of the boundaries of a jet plume in its flight maneuvers. A comparison of initial zigzagging in narrow and wide jet plumes may show a relationship between plume width and counterturn position. At this point we must assume that straight flight and recurrent zigzagging are more or less fixed and not affected by plume width, because no difference between treatments was demonstrated.

Distance traveled in each square together with the plume width is plotted in Figure 9B. Wasps flew significantly greater distances in narrow plumes as compared to wide plumes ($P < 0.0003$). This might be due to more frequent counterturning because the maximum flight deviation from the center line did not differ among treatments. Dilution of the odor load in each size jet plume, wide or narrow, did not significantly affect the distance traveled. When differences between narrow and wide plumes were tested for each square separately, the differences were significant for squares 5, 6, and 7 ($P < 0.0278, 0.0447, 0.0122$). The differences in maneuvers above squares 5, 6, and 7 of wide and narrow jet plumes are due to the fact that in narrow plumes initial zigzagging was performed upwind from the release point, whereas in wide plumes parasitoids more often performed straight flight. Above the more upwind squares 1, 2, 3, and 4, straight flight and recurrent zigzagging were of similar frequency for both jet plumes.

The center line air velocity of jet plumes was plotted with the ground speeds of the wasps for each square (Figure 9C). In a wide jet plume, the ground speed decreased toward the source from 30 to 23 cm/sec; however, this decline was not significant. Consequently the airspeed varied between 80 and 85 cm/sec close to the release point and between 73 and 113 cm/sec 10 cm downwind from the target. Although not significant, the average ground speed in narrow low-velocity plumes decreased from around 23 to 18 cm/sec. This decrease could not result from exceeding demands on airspeed by the jet plume, because *M. croceipes* can fly at least 113 cm/sec as was observed in the wide high-velocity plume, and the maximum demand in a narrow plume was 71 cm/sec 10 cm downwind from the target. Females flew at significantly lower ground speeds in narrow jet plumes compared to wide jet plumes ($P < 0.0001$). This

can partly be explained by the assumption that the initial flight stage is at slower ground speed.

DISCUSSION

The orientation mechanisms used during in-flight host searching by parasitoid wasps of herbivorous larval insects are not well understood. Traditionally the first two steps of host searching in parasitoids are called host habitat location and host location. *Heliothis* has a polyphagous feeding pattern and switches among host plants during the season. Several host-related olfactory cues might be used during habitat and host location. The relative importance of each of these cues for host location depends on how closely it is associated with the presence of the host. From this perspective, cues that persist in attracting *M. croceipes* after the larva moved elsewhere may be less important for immediate host detection, whereas semiochemicals exclusively emitted by larvae are more valuable. Odors emitted by the larva itself or odors with little persistence and emitted during feeding fall into this class. The odors used by *M. croceipes* for detection of hosts come from sources that may differ in strength. In our set-up, only the strongest sources associated with the host-plant complex might have been active. The use of an extensive odor supply system was intended to stabilize odor emission, shape of odor plume, and visual stimuli at the source. The experiments presented in this paper use an indirect indicator for emission rates of semiochemicals: feeding activity of the host larvae.

Female *M. croceipes* respond to semiochemical sources that are similar to those evoking pheromone-guided responses by male moths. In both cases the semiochemicals emanate from point sources, although the location of the sources on the foliage may differ. In both cases the insects involved are fast and strong flyers that are able to approach the source from downwind. The olfactory cues are emitted by the objects that evoke the consummatory act, which are, respectively, egg deposition into the host larva and copulation with a "calling" female moth. In both cases intraspecific competition might have led to optimization of flight maneuvers to enhance searching success.

However, in larval parasitoids interspecific interaction might have increased the intensity of competition. *M. croceipes*, for example, competes with other entomophagous insects for *H. zea* larvae. Another factor that cannot be ignored is the possibility of defensive actions by the attacked host. Our experiments excluded interference of defensive behavior of the host with orientation behavior of the parasitoids.

The question arises as to what caused oriented flights between feeding bouts of the host. Sustained flights were not observed by Drost et al. (1986) to undamaged cowpea seedling leaves in a flight tunnel or by Eller et al. (1988)

to volatiles emitted by isolated *H. zea* larvae in an airflow olfactometer as described by Vet et al. (1983). Fecal pellets and artificially damaged cowpea seedling leaves attract female parasitoids in an airflow olfactometer (Eller et al., 1988). The emission of volatiles from plant damage and possibly host secretions associated with feeding is probably short-lasting, because Eller et al. (1988) found that collected odors from hosts feeding on cowpeas were behaviorally inactive after 10 min. In our experiment, the median period between feeding bouts for a group of three starved fifth-instar *H. zea* was 5 min, and therefore we probably did not measure the effect of fading of the source. In addition, volatiles rereleased from the glass walls might have caused sustained flights between feeding bouts.

It is surprising that the percentage of sustained flights in narrow plumes with a half odor load was less than in a wide plume with a full odor load (Figure 6A). Response would not be expected to differ when odor concentrations are of the same order of magnitude (Figure 8). Because only a minority of all non-successful responses were temporary-oriented flights, *M. croceipes* evidently will not interrupt its flight due to increasing odor concentrations. In moths, increasing pheromone concentrations typically decrease net upwind speed (e.g., Cardé and Hagaman, 1979; Sanders, 1985).

In moths attracted to pheromone, an internal program of counterturns (zig-zags) is thought to be evoked by pheromones (Cardé, 1984; Kennedy, 1983; but see also Preiss and Kramer 1986). The rate of upwind displacement is modulated by odor concentration, causing a decrease of net upwind speed close to the source, resulting from a combination of an increased frequency of zigzagging and a decrease in flight speed. Fluctuations in the odor concentration are needed to maintain counterturn responses in *Grapholitha molesta* (Baker et al., 1985). Wind experienced prior to take-off or while in flight establishes polarity of the counterturns, so that displacement toward the source will occur. Thus odor concentration modifies the anemotactically polarized optomotor responses performed by moths.

One experimental method to study concentration effects on oriented flights is the use of moving floor patterns in a flight tunnel. The effect of the floor pattern being moved in the downwind direction by the experimenter will be that the responding insect will lower its flight speed. Once the floor movement is at a level equal to the "preferred" retinal velocity, the insect will have lowered its flight speed to a level where it remains at a relatively fixed position in the flight tunnel. One can thus determine how odor concentration alters the optical feedback needed to arrest upwind progress of a flying insect (Sanders, 1985). The advantage of this method over using a jet plume is that substantially less wind shear can be expected as compared to jet plumes. In jet plumes air velocity gradients are accompanied by odor concentration gradients.

An insect able to perceive its acceleration can determine wind direction in a jet plume without using optical information, following principles proposed by Gillett (1979). When flying upwind with a fixed airspeed, an insect will decelerate at the center of the jet plume, whereas it will accelerate when shifting its course away from the center of the jet plume. In a downwind heading, similar maneuvers will have opposite effects on flight velocity. Therefore one cannot determine if insects flying in jet odor plumes use either optical or inertial input to maintain an upwind course. Another important difference between jet plumes and a regular point source is their distribution of odors. Murlis and Jones (1981) concluded that odor plumes emerging from point sources do not contain information that enables insects to determine their distance relative to the source. For this reason, previous studies of concentration effects have been possible only by using point sources with different odor emission rates. In a jet plume one can manipulate upwind odor gradients by changing the dimensions of the jet using the same source. In moths, upwind progress decreases around three-fold with a 10-fold increase of odor load of the test sources (Cardé and Hagam, 1979; Sanders, 1985). In contrast, *M. croceipes* does not respond to odor concentration in this manner. Ground speed was not affected by increasing odor concentrations over a 20- to 67-fold range as the wasp progressed upwind in jet plumes. The fact that *M. croceipes* responds with increasing flight speeds to increasing odor concentrations is the opposite of the response found to date with moths.

The stereotypic shift from initial zigzagging to straight flight and the evidently minor importance of odor concentrations or odor fluxes in modulating the upwind progress may be explained as an adaptation to short communication distances and unstable odor sources. *M. croceipes* interrupted straight upwind flight with two types of maneuvers: recurrent zigzagging and downwind loops. Downwind loops were predominant in irregularly shaped plumes. In jet plumes, recurrent zigzagging was most often performed. Loss of contact with the odor stimulus can result from windshifts and interruption of odor emission. *M. croceipes* hunting for third-instar larvae can expect larval feeding activity only 50% of the time, as was found by Schmidt (1985). David et al. (1983) describe how wide lateral zigzags aid the gypsy moth in reentering a plume "lost" because of shift in wind direction. His model is applicable when windshifts are the main cause of scent loss and interruption of odor emission is unlikely. When interruptions of odor emission occur, which may be the case in *M. croceipes*, a strategy other than casting may be more effective in enhancing the likelihood of recontacting the plume. All course angles that are crosswind but have no upwind component are more effective in finding another source, because for a given amount of flight, the area searched is greater than for course angles with an upwind component. Both casting and downwind loops thus may serve as a

strategy for recontacting a lost scent over the distances of host detection used by *M. croceipes*.

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EFFECT OF ERGOT ALKALOIDS FROM FUNGAL ENDOPHYTE-INFECTED GRASSES ON FALL ARMYWORM (*Spodoptera frugiperda*)

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Abstract—Ergot alkaloids produced by endophytic fungi in the tribe Balansiae (Clavicipitaceae, Ascomycetes), which infect grasses, may provide plant defense against herbivores. This study examined the effects of six ergot alkaloids on survivorship, feeding, and growth of larvae of the fall armyworm (*Spodoptera frugiperda*, Lepidoptera: Noctuidae), a generalist herbivore of grasses. Corn leaf disks were soaked in solutions of individual ergot alkaloids at different concentrations and presented to neonate larvae. At the highest concentrations (77–100 mg/liter) of ergonovine, ergotamine, ergocryptine, agroclavine, and elymoclavine, larval weights and/or leaf area consumed after eight days were reduced relative to controls. Lysergol had no effect on larval weights and leaf consumption at any concentration. Although active concentrations were higher than those reported from two host grasses, *in vivo* levels of ergot alkaloids have not been quantified for most endophyte-infected grasses. The detrimental effects on fall armyworm observed in this study suggest that ergot alkaloids could be responsible, at least in part, for the greater insect resistance of endophyte-infected grasses.

Key Words—Balansiae, Clavicipitaceae, ergot alkaloids, fall armyworm, feeding deterrents, acquired chemical defense, fungal endophytes, grasses, Lepidoptera, Noctuidae, *Spodoptera frugiperda*.

INTRODUCTION

Recently the role of clavicipitaceous fungal endophytes infecting grasses as insect feeding deterrents has been demonstrated (Funk et al., 1983; Clay et al., 1985; Latch et al., 1985). Systemic fungi in the tribe Balansiae, family clavi-

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cupitaceae (Ascomycetes), and their anamorphs, are known to infect hundreds of species of grasses and sedges (Diehl, 1950; Clay, 1987; White, 1987). Livestock toxicity problems of unknown etiology historically have been associated with certain pasture grasses (e.g., tall fescue, perennial ryegrass). The presence of fungal endophytes in these grasses has been correlated with livestock toxicity (Bacon et al., 1986). Perennial ryegrass pastures in New Zealand toxic to sheep were shown also to be more resistant to damage by the Argentine stem weevil, *Listronotus bonariensis* (Prestidge et al., 1982). The association between fungal endophyte infection and resistance to insect herbivores has now been demonstrated in a number of grasses and sedges (Clay, 1987).

Increased insect resistance of infected grasses may result from the production of alkaloids by the fungi present within the tissues of its host. The Balansiae, like their close relatives in the genus *Claviceps*, produce a variety of ergot alkaloids. However, unlike *Claviceps*, which is a localized ovarian parasite of grass flowers, Balansiae species are systemic, and both the fungi and the alkaloids can be found throughout the above-ground host-plant tissues (Clay, 1986; Siegel et al., 1987). The anamorphic endophytes of tall fescue and ryegrass and several Balansiae species are also known to produce other types of alkaloids (Porter et al., 1977; Bush et al., 1982; Gallagher et al., 1984; Rowan and Gaynor, 1986).

While the association among fungal endophytes, ergot alkaloids, and insect resistance in grasses strongly suggests a functional relationship, to our knowledge there have been no previous published studies of the effect of ergot alkaloids on insect feeding. The purpose of this study is to determine the effect of several ergot alkaloids on feeding by larvae of the fall armyworm (*Spodoptera frugiperda* J.E. Smith, Lepidoptera: Noctuidae), a generalist herbivore of grasses. The fall armyworm has been shown previously to be sensitive to the presence of fungal endophytes in grasses (Clay et al., 1985; Hardy et al., 1985, 1986), and it has been widely utilized as a bioassay for grass feeding studies (Pencoe and Martin, 1981). In particular, we ask whether the alkaloids function as antifeedants and/or antibiotics and whether specific alkaloids representing the major classes of ergot alkaloids differ in their toxicity.

METHODS AND MATERIALS

The Alkaloids. A large number of ergot alkaloids have been isolated from Balansiae fungi-infected grasses or extracted from fungal cultures (Bacon et al., 1979; Porter et al., 1979; Lyons et al., 1986). In general, ergot alkaloids are characterized by the ergoline nucleus (Cordell, 1981) (Figure 1) and can be divided into three general groups: clavine alkaloids, lysergic acid alkaloids, and ergopeptide alkaloids (Cordell, 1981). Clavine alkaloids have a $-\text{CH}_2\text{OH}$ or

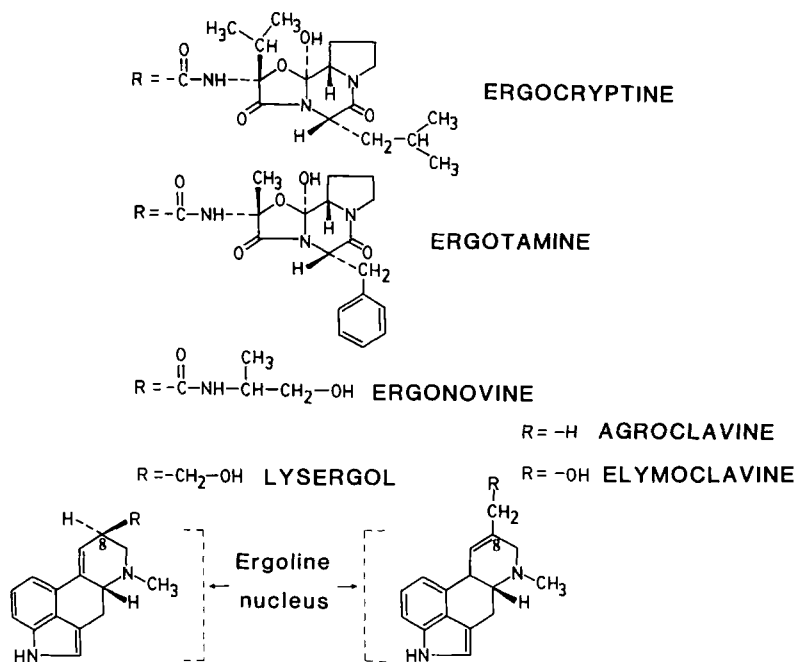


FIG. 1. Structure of the six ergot alkaloids utilized in this study. An 8 denotes the C-8 position of the ergoline nucleus.

—CH₃ group at the C-8 position while lysergic acid alkaloids have a carboxyl or carboxamide group at that position; ergopeptide alkaloids are amides of lysergic acid with dipeptide or tripeptide structures (see Figure 1) (Waller and Dermer, 1981). The alkaloids utilized in this study included members of each of these types (agroclavine, elymoclavine, and lysergol—clavine types; ergonovine—lysergic acid type; ergotamine and ergocryptine—ergopeptide type) (Cordell, 1981). The structures of these compounds are illustrated in Figure 1. Agroclavine and elymoclavine were supplied by Eli Lilly and Co., Indianapolis, Indiana while the remaining alkaloids were obtained commercially from Sigma Chemical Co., St. Louis, Missouri. Agroclavine, elymoclavine, and ergonovine have been reported from *Balansia* and *Epichloë* species (Bacon et al., 1979; Porter et al., 1981), and ergotamine has been isolated from pure cultures of *Atkinsonella hypoxylon* (unpublished), all members of the Balansiae.

Bioassay. Aqueous solutions of ergonovine and ergotamine were prepared at the following concentrations: 0, 0.077, 0.77, 7.7, and 77 mg/liter. A few drops of 0.0001% Tween 80 were added to each to facilitate adherence of the solution to fresh plant material. This provided an evenly distributed layer of

solution across the disk surface: the weight of water deposited onto a single 1-cm-diameter disk using this method was 16.8 ± 1.3 mg ($\bar{X} \pm \text{SE}$; $N = 10$). Corn (*Zea mays* L., variety Illini-Xtra Sweet) leaves were used in all feeding trials because corn is known to be a preferred food of the fall armyworm, the tissues do not contain ergot alkaloids, and the wide leaves are suitable for obtaining leaf disks of known area. Leaf disks were soaked in each alkaloid solution for 3 min and then put onto pins until all water had evaporated (ca. 10 min). Leaf disks then were placed individually into plastic Petri plates lined with moistened filter paper and newly emerged larvae of the fall armyworm were immediately added, one larva per disk per Petri plate. There were 30 Petri plates per alkaloid solution. Petri plates were kept in transparent plastic bags to maintain high humidity and stored in an incubator with a 16 hr light-8 hr dark, 25°C-20°C diurnal cycle.

The alkaloid concentrations chosen were based on data from endophyte-infected tall fescue presented in Lyons et al. (1986), where ergot alkaloid concentrations ranged from less than 0.1 mg/kg plant tissue to over 10 mg/kg with an average of about 1 mg/kg. Based on the mean dry weight per 1 cm corn leaf disk (1.3 mg) and the mean weight of water deposited onto each disk (16.8 mg) by our methods, the intermediate concentration we used (0.77 mg/liter) approximated mean field levels of ergot alkaloids in tall fescue. The other concentration levels provided a convenient range in 10-fold increments to encompass and exceed the variability in ergot alkaloid content found in endophyte-infected plants (Lyons et al., 1986). Because most of the effects of ergonovine and ergotamine, the first alkaloids screened in this study, were detected at the highest concentrations, for the remaining four alkaloids an expanded range of concentrations was used: 0, 1, 10, and 100 mg/liter.

Every three days, the leaf disks were removed and the unconsumed leaf area remaining was determined with a LI-3100 leaf area meter (Li-Cor, Inc., Lincoln, Nebraska). Fresh leaf disks were added to each plate at this time. The experiment was terminated after eight days. Fall armyworm larval weights were then obtained, and total leaf area consumed was determined by subtracting the unconsumed leaf area from the total leaf area originally presented to the larvae.

Determination of Alkaloid Deposition. To quantify the rate and variation in alkaloid deposition onto the corn leaf disks, an experiment was performed using ergonovine. Ten corn leaf disks were soaked in an ergonovine solution (1 g/liter) as described above. After the disks had dried, they were cut into quarters and the alkaloid deposited onto each piece was quantified colorimetrically following Michelin and Kelleher (1963). Each leaf piece was agitated in 2 ml of a 2% tartaric acid solution. After removing the leaf piece, 2 ml of 0.1% PDAB (paradimethylaminobenzaldehyde) in sulfuric acid-water (1:1 v/v) was added to the 2 ml tartaric acid containing dissolved alkaloid. The solution was mixed well: after 10 min, 0.1 ml of 0.1% aqueous sodium nitrite was added to each solution. Following color change, absorbance was measured with

a spectrophotometer (Turner model 350) at 590 nm. Alkaloid content was calculated from a regression equation based on absorbances known from a prior calibration using a range of concentrations of ergonovine solutions. The high concentration of ergonovine used was necessitated by the sensitivity of this technique.

Analyses. For each alkaloid, *G* tests of independence were used to test differences in larval survival between the control (0 mg alkaloid) and the other concentrations (Sokal and Rohlf, 1981). One-way analysis of variance was used to determine whether leaf area consumed and larval weights differed among concentration levels within an alkaloid, and if so, least significant differences were used to compare means (SAS Institute, 1985). Log transformations were used to improve normality when necessary. To examine the relationship between leaf area consumed and larval weight, and specifically whether alkaloid concentration affected this relationship, linear regressions were performed using weight as the dependent variable. Regression coefficients (slopes) for each alkaloid concentration level were compared to the control by the Tukey-Kramer method for comparing regression lines (Sokal and Rohlf, 1981).

RESULTS

Bioassay. For ergonovine and ergotamine, larval survival was uniformly high across all alkaloid concentration levels (Table 1), ranging from 83% to 97%. Larval survival tended to be slightly lower at high concentrations for the other alkaloids, but was only significantly lower than the control for ergocryptine at 100 mg/liter and elymoclavine at 10 mg/liter (Table 1).

Although total leaf disk consumption did not vary significantly among different concentrations of ergonovine (Table 2), larval weights were significantly

TABLE 1. SURVIVAL (%) OF FALL ARMYWORM LARVAE FEEDING FOR EIGHT DAYS ON CORN LEAF DISKS SOAKED IN VARIOUS SOLUTIONS OF ERGOT ALKALOIDS^a

Alkaloid	Concentration (mg/liter)							
	0	0.077	0.77	1	7.7	10	77	100
Ergonovine	93.3	86.7	93.3		96.7		96.7	
Ergotamine	93.3	93.3	96.7		93.3		83.3	
Ergocryptine	83.3			83.3		90.0		46.7**
Lysergol	93.3			86.7		86.7		83.3
Agroclavine	83.3			86.7		73.3		76.7
Elymoclavine	93.3			80.0		70.0* ^b		83.3

^a*N* = 30 for each alkaloid at all concentrations.

^b*P* < 0.05, ***P* < 0.01; *G* test of independence comparison with 0 concentration.

TABLE 2. RESULTS OF ONE-WAY ANOVA FOR LARVAL WEIGHT AND TOTAL LEAF AREA CONSUMED AFTER EIGHT DAYS FOR EACH ALKALOID

Alkaloid	Source of variation	df	Larval weight			Leaf area consumed		
			MS	F	P <	MS	F	P <
Ergonovine	conc.	4	0.41 ^a	2.24 ^a	0.068	2.57	0.71	0.588
	error	135	0.18 ^a			3.62		
Ergotamine	conc.	4	442.05	12.32	0.001	42.50	15.19	0.001
	error	133	35.87			2.80		
Ergocryptine	conc.	3	221.60	2.98	0.036	17.11	2.45	0.069
	error	87	74.48			6.97		
Lysergol	conc.	3	9.81	0.13	0.939	1.91	0.25	0.823
	error	101	72.93			7.71		
Agroclavine	conc.	3	198.69	5.17	0.002	16.53	5.26	0.002
	error	92	38.43			3.14		
Elymoclavine	conc.	3	125.19	6.57	0.001	8.90	3.98	0.010
	error	94	19.05			2.24		

^aLog-transformed data.

lower than the control at the highest concentration (Figure 2A). This suggests that ergonovine acted as an antibiotic rather than as an antifeedant. Further evidence is presented in Figure 3, where larval weight is plotted against leaf area consumed. The slopes of these regression lines are significantly lower than the control at 0.77 and 77 mg/liter ($P < 0.05$), although not at 7.7 mg/liter, indicating that for any particular level of consumption, larvae gained less weight at these concentrations of ergonovine when compared to the control which contained no alkaloid.

In contrast to ergonovine, leaf disk consumption was significantly reduced at the highest concentration of ergotamine (Table 2, Figure 2B). In addition, the slope of the regression line for larval weight vs. consumption at this concentration (77 mg/liter) was significantly lower than the control ($\bar{X} \pm SE = 2.85 \pm 0.18$ and 3.60 ± 0.15 , respectively; $P < 0.05$). Larval weights were also lowest at this concentration; however, at intermediate alkaloid levels, weights were higher than those of the control (Figure 2B), even though leaf consumption was not significantly greater than the control at the 0.077 and 0.77 mg/liter concentrations.

The pattern of heavier larval weights despite no significant increase in consumption at intermediate alkaloid levels was also evident for ergocryptine (Figure 4A). Again, both larval weight and leaf consumption were reduced at the highest concentration. Slopes of the regression lines for larval weight vs. con-

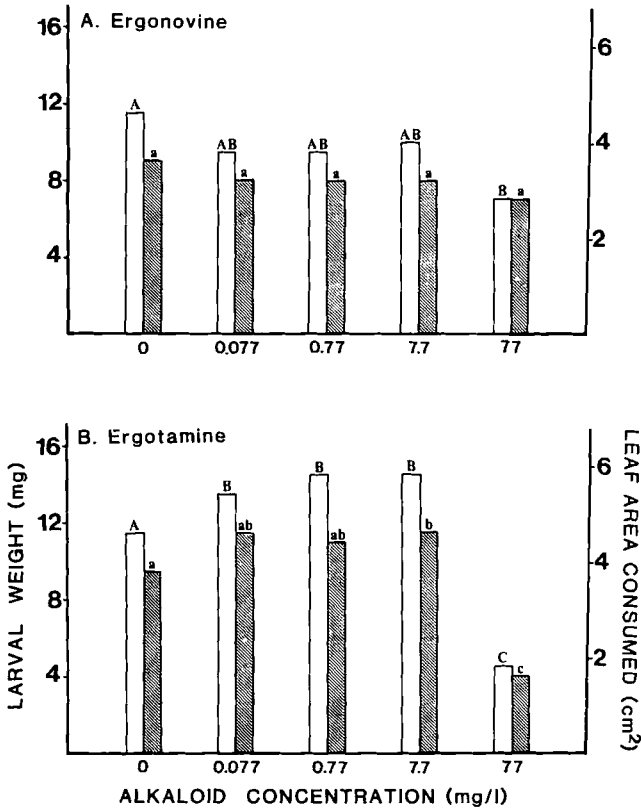


FIG. 2. Effect of various concentrations of two ergot alkaloids, (A) ergonovine and (B) ergotamine, on eight-day larval weight (open bars) and leaf area consumed (hatched bars). Means with the same capital letter (open bars) or with the same lower case letter (hatched bars) are not significantly different ($P > 0.05$).

sumption at all concentrations did not differ from that of the control, suggesting that ergocryptine acts as an antifeedant to fall armyworm larvae.

The three clavine alkaloids differed greatly in their effects on the fall armyworm. Lysergol had no effect on larval consumption or weight at any concentration, and resulted in the heaviest larvae of any of the alkaloid feeding trials (Table 2, Figure 4B). However, highly significant effects were detected for agroclavine and elymoclavine (Table 2). For both alkaloids, leaf consumption and larval weight were lowest at the highest alkaloid concentration (Figures 4C and D). In addition, the slopes of the regression lines for larval weight vs. consumption were significantly lower than the control (3.67 ± 0.19) at 1 (2.99

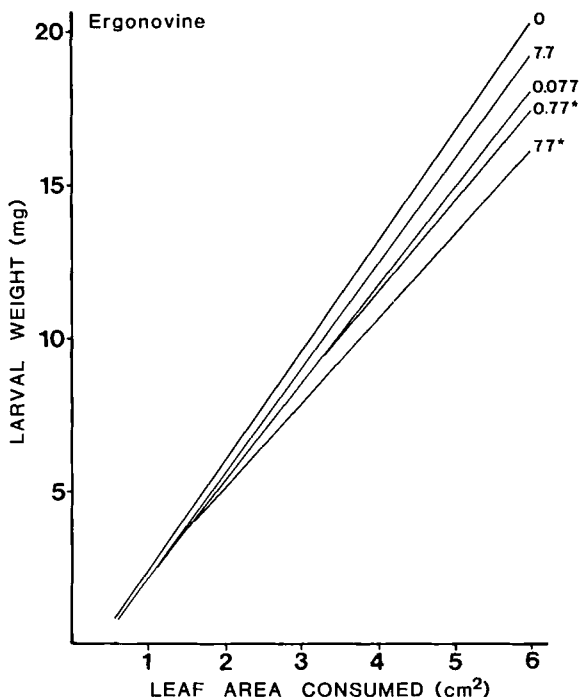


FIG. 3. Relationship between leaf area consumed and larval weight at various concentrations of ergonovine. Asterisks indicate lines with slopes significantly lower than the control ($P < 0.05$).

± 0.17) and 100 mg/liter (3.16 ± 0.17) concentrations of agroclavine ($P < 0.05$). Like ergocryptine, elymoclavine did not show these differences among slopes; it presumably acts primarily as an antifeedant at high concentration (Figure 4D).

Alkaloid Deposition. Ergonovine was detected on every piece from each leaf disk. Based on leaf area, amount of solution absorbed, and alkaloid concentration, 7.17 μg ergonovine was expected from each quarter leaf piece. A mean of 6.78 μg was determined (± 0.58 SE), a recovery rate of 95%. Although there was variation among pieces in deposition rate (range 1.47–14.79 μg), 30 of 40 leaf disk quarters had values between 3 and 10 μg . Moreover, there were no significant differences among leaf disks in total alkaloid deposition. Thus, these results indicate that the technique of soaking leaf disks in alkaloid solutions resulted in a fairly uniform delivery of alkaloid to the insect larvae.

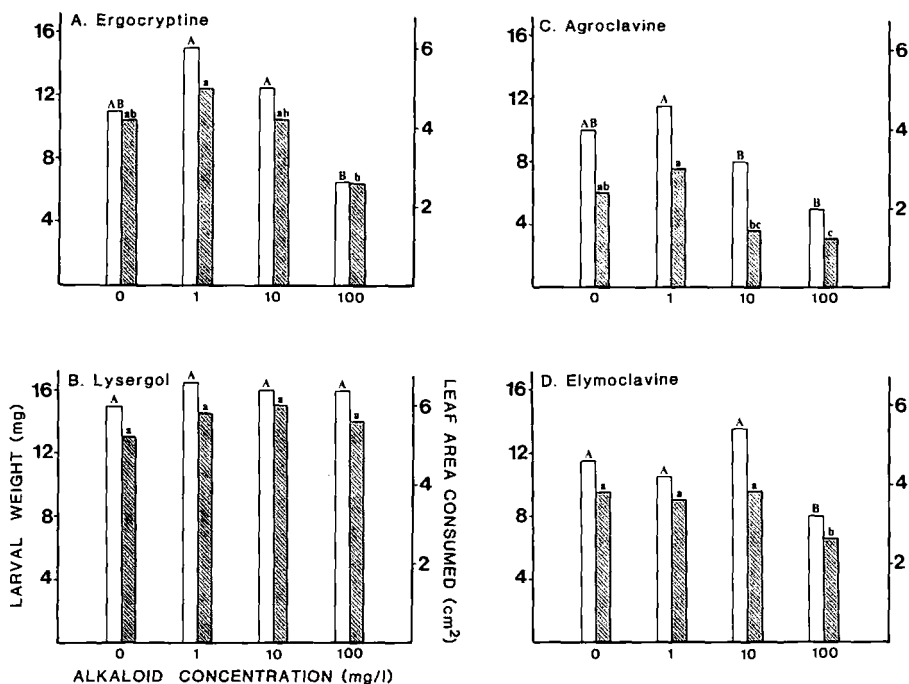


FIG. 4. Effect of various concentrations of four ergot alkaloids: (A) ergocryptine, (B) lysergol, (C) agroclavine, and (D) elymoclavine, on eight-day larval weight (open bars) and leaf area consumed (hatched bars). Means with the same capital letter (open bars) or with the same lower case letter (hatched bars) are not significantly different ($P > 0.05$).

DISCUSSION

With the exception of lysergol, the ergot alkaloids examined in this study affected fall armyworm larvae feeding on corn leaf disks. In contrast, the alkaloids had little effect on larval survival; only two of 20 comparisons were significantly lower than the control. At the highest alkaloid concentrations, there were significant and dramatic declines in both larval weights and leaf consumption. At these concentrations the alkaloids appeared to function as antibiotics (ergonovine), antifeedants (ergocryptine and elymoclavine), or both (ergotamine and agroclavine) in relation to their effects on the fall armyworm.

There appeared to be little relationship between the structural features of the different alkaloids and their effects on fall armyworm larvae. For example,

there were highly significant differences among concentrations of agroclavine and elymoclavine on both larval weights and leaf consumption, while lysergol, also a clavine alkaloid with a similar structure (Figure 1), had no effect on fall armyworm (Table 2). There was no evidence that ergopeptide alkaloids were any more toxic than the clavine alkaloids in this study, although they are suspected to be more toxic to mammalian herbivores (Bacon et al., 1986).

Somewhat surprisingly, at lower concentrations, several of the alkaloids resulted in increased larval weights and leaf area consumption compared to the control, although the differences were significant only for ergotamine (Figure 2B). While alkaloids are nitrogen-rich compounds, it is unlikely that they contributed significantly to the nitrogen budget of the larvae. For example, a 1-cm corn leaf disk (1.3 mg mean dry weight) contains approximately 52 μg N [Schrader et al. (1972) indicated that corn leaves contain on average about 4% N on a dry weight basis.] At the highest concentration of ergonovine, with our technique approximately 1.3 μg alkaloid would be deposited containing 0.17 μg N. Although the reason for the initial increase in larval weights at low alkaloid concentrations is unclear, similar phenomena have been documented in other insects. Reese (1979) discussed several cases where insecticides at low concentrations actually stimulated insect growth. Similarly, Maxwell et al. (1967) reported that low levels of gossypol from cotton stimulated feeding and oviposition of the boll weevil but inhibited them at high concentrations.

The effect of ergot alkaloids on herbivores has been well documented for mammalian herbivores grazing *Claviceps*-infected grasses (Mantle, 1969) and for human populations consuming ergot-infested grain products (Bové, 1970; Groger, 1972). However, in *Claviceps* spp. the alkaloids are concentrated in the sclerotium, upon which few insects feed. Grasses systematically infected by Balansiae species contain fungal alkaloids within plant tissues and can be toxic to both mammalian and insect herbivores (Bacon et al., 1986; Clay, 1986). Mammalian toxicity resulting from the consumption of Balansiae-infected grasses resembles ergotism, suggesting that the causal agent is the same, i.e., ergot alkaloids (Bacon et al., 1975; Porter et al., 1981). The chemical basis for insect feeding deterrence and antibiosis has not been shown definitively for Balansiae-infected grasses, although in perennial ryegrass and tall fescue infected by anamorphic *Acremonium* spp., peramine and pyrrolizidine alkaloids, respectively, are known to be feeding deterrents for some insects (Johnson et al., 1985; Rowan and Gaynor, 1986). The presence or absence of these compounds in grasses infected by *Balansia* and other related fungi has not been reported in the literature. In contrast, ergot alkaloids occur in Balansiae-infected grasses and are produced by fungal cultures, and a number of infected grasses have been shown to be resistant to fall armyworm (Bacon et al., 1979; Clay et al., 1985). Here we have shown that fall armyworm larval weights and/or feed-

ing on corn leaf disks is reduced at high concentrations of several ergot alkaloids.

Some of the variability observed in this study may have resulted from differential deposition of alkaloids onto the leaf disks. In the deposition experiment with ergonovine, there were no significant differences among leaf disks in overall alkaloid level, although there was some variability in deposition on different parts of the disk. Larvae may have preferentially fed upon areas of the disk with slightly lower concentrations; nevertheless, minor variations among leaf disks at the same concentrations should have no effect on the pattern of mean response because leaf disks used at all alkaloid levels and controls were treated in the same manner. However, in light of the variability in deposition, the statistically significant differences detected in this study are conservative.

The greatest antifeedant and antibiosis effects were achieved in this study at alkaloid concentrations of 77–100 mg/liter. These concentrations correspond to values of between 1 and 1.3 g/kg plant tissue, which is approximately two orders of magnitude higher than the concentrations of ergot alkaloids reported for endophyte-infected tall fescue and *Balansia epichloë*-infected smutgrass (Bacon et al., 1979; Lyons et al., 1986). It is possible that the ergot alkaloids, at the levels reported in tall fescue, play only a minor role in the observed resistance of endophyte-infected plants to insect herbivores (Clay et al., 1985; Hardy et al., 1986). Although the endophyte of tall fescue also produces pyrrolizidine alkaloids, Hardy et al. (1986) found that the youngest leaves contained the highest concentration of nearly 1000 mg/kg dry weight leaf tissue but the greatest growth of armyworm larvae occurred on the youngest leaves, suggesting that these alkaloids are not the primary feeding deterrent. In feeding studies of fall armyworm larvae there were significant decreases in larval weight gain and rate of development when reared on infected tall fescue compared to uninfected tall fescue (Clay et al., 1985; Hardy et al., 1986). However, other endophyte-infected grasses elicited much more dramatic responses in the same insect, suggesting that they were more toxic, perhaps containing higher levels of ergot alkaloids (Clay et al., 1985; Cheplick and Clay, 1988). With the exception of smutgrass, which has not been subject to insect feeding studies, ergot alkaloid levels in other endophyte-infected grasses have not been quantified.

The role of alkaloids in plants has been subject to debate, but many researchers agree that alkaloids often play a defensive role, protecting plants against herbivores (Fraenkel, 1959; Freeland and Janzen, 1974; Levin, 1976; Robinson, 1979). There is a vast literature on the effects of plant secondary compounds from various plants on different insects. Patterns of host specificity of particular groups of insects indicate that secondary compounds have been an important selective force in the coevolution of plants with their insect herbivores. The widespread relationship between grasses and endophytic fungi, and

their effect on herbivores, suggests that ergot alkaloids may be playing a similar role to alkaloids found in other plant families by providing protection against herbivores. Our data are in agreement with the hypothesis that ergot alkaloids can act as insect feeding deterrents. In contrast with the direct biosynthesis of defensive compounds by plant tissues in many plant taxa, endophyte-infected grasses represent a special case of acquired chemical defense against herbivores (Cheplick and Clay, 1988). The secondary products of endophyte metabolism could partially compensate for the general lack of insect-detering compounds in the grass family.

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COUNTERTURNS INITIATED BY DECREASE IN RATE OF INCREASE OF CONCENTRATION

Possible Mechanism of Chemotaxis by Walking Female *Ips paraconfusus* Bark Beetles

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Abstract—The position of beetles were marked at 1-sec intervals after they were released in still air 16–18 cm from point sources of pheromone. Characteristics of the tracks were quantified and compared to those that might be produced by counterturning schemakinesis, tropotaxis, klinotaxis, zigzagging, look-and-leap, or steepest-ascent schemakinesis mechanisms. The beetles' movements were highly irregular, but they turned almost continually and never fixed on a heading near 0° (=straight towards the source). Turn angle sizes increased slightly with absolute size of heading but had the opposite sign, thus compensating slightly for heading. Their distribution was centered about 0° and was unimodal. Heading decreased gradually as the source was neared, but the decrease became steeper within 1–5 cm of the source. Histograms showed that the maximum headings between occurrences when the beetle was headed directly towards the source (0°) were centered around 0° and most of them were less than 90°. However, maximum headings between 90° and 180° were not uncommon. Turn radius decreased as the source was neared. The counterturning mechanism was the most consistent with these observations. An analysis of rate of change of concentration with respect to heading and distance to the source further demonstrated that the counterturning mechanism could explain the form of the decrease in heading as the source was neared, if the major cue used to initiate counterturns was a decrease in the rate of increase of concentration. The tropotaxis could not recreate the form of the decrease, under any form of stimulus processing.

Key Words—*Ips paraconfusus*, Coleoptera, Scolytidae, bark beetles, olfaction, orientation, counterturning, chemotaxis, taxis, pheromones, tropotaxis, schemakinesis.

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INTRODUCTION

Ips paraconfusus Lanier (Coleoptera: Scolytidae) has three compounds in its pheromone, which it uses to orient with respect to mates, competitors, and hosts (Silverstein et al., 1966; Wood et al., 1967). The analyses described here arose from experiments undertaken to develop a behavioral assay for olfactory quality discrimination among the components of the pheromone (Akers, 1985; Akers and Wood, 1987b). Chemotaxis was one behavior used as the basis for an assay. The responses in this assay were striking (Akers, 1985; Akers and Wood, 1987a). From a distance of 16–18 cm, approximately 80–90% of all beetles located sources of 0.1–1.0 mg of pheromone, guided by odor alone. I became interested in the mechanisms underlying the taxis, because of its apparent efficiency. A more complete description of the mechanism was not necessary to use the assay as an indicator of olfactory quality, but it would aid in limiting the possible neural mechanisms that could be involved in orientation.

Previous work established the hypothesis that the initiation of chemotaxis was essentially an all-or-none phenomenon (Akers, 1985; Akers and Wood, 1987a). The behavior of beetles that did and did not reach the source was qualitatively different, while the behavior of beetles within either of these groups was very similar, even though the beetles were exposed to dosages differing by several orders of magnitude. Thus, the major influence of increasing pheromone dosage was to increase the probability that a beetle would initiate orientation.

The mechanisms by which an orienting beetle reaches the odor source are explored in this paper. Its premise is that orientation based on different sources of information will produce tracks that differ in form. Quantitative analyses were designed to describe various aspects of track form. The predicted effect of several possible orientation mechanisms on each analysis was then compared to the observed results to identify any particular mechanism that might be consistent with the observations. The analysis indicated two major hypotheses concerning the mechanism of orientation. First, an orienting beetle locates the source by turning in one direction until it detects a critical sensory cue, at which time it initiates a turn in the opposite direction. This mechanism is termed a counterturning schemakinesis (Kennedy, 1986), or counterturning, for short. Second, if counterturning is the mechanism, the major cue used to initiate a counterturn appears to be a decrease in the rate of increase of concentration.

METHODS AND MATERIALS

The compounds used as stimuli, the observation chamber, the recording procedures, the handling of the experimental animals, the experimental design, and some of the analytical methods for orientation data have all been described

previously (Akers and Wood, 1989a). The basic experimental design was a dosage series of a 1 : 1 : 1 blend of the three pheromonal compounds, ipsenol (Ip), ipsdienol (Id), and *cis*-verbenol (cV). The dosages differed by powers of ten, running from 10^{-4} to 1 mg, and were dissolved in 0.1 ml pentane. The series was run with diffusion periods of 30 and 60 min in separate experiments.

The position of each beetle was marked at 1-sec intervals after it was released in still air 16–18 cm from a point source of pheromone. The angle the beetle turned at each point on its track was measured. The turn angle was defined as the angle between the direction from the current point had the beetle continued walking straight ahead along the line from the previous to the current point on the track, and the direction from the current to the next point. Zero degrees was defined as straight ahead. A heading was also measured at each point on a track. The heading is the direction an animal takes with respect to the pheromone source, at any given point on its track. It was defined as the angle between the direction towards the source from the current point on the track and the direction from the current point to the next point on the track. Zero degrees was defined as straight towards the source. For “net” angle summaries, left-hand angles were defined as positive. For “gross” angle summaries, the absolute values of the angles were used (Akers and Wood, 1989a). Summaries of walking rate and its variation within a track, net turning rate, gross turning rate and its variation within a track, mean net heading, and gross heading and its variation within a track have been reported elsewhere (Akers, 1985; Akers and Wood, 1989a). Further analyses were needed to identify the probable mechanism of chemotactic orientation. Unless otherwise noted, the following analyses were made on only those beetles in a treatment that reached the source and on control beetles when comparisons with nonorienting beetles seemed appropriate.

Histograms were made of the size of the turn angles, to determine whether their distribution was unimodal or bimodal. A bimodal distribution would have indicated that the beetles had two distinct rates of turning, such as might occur if linear sections of track were interspersed with more rapid turning. A bin size of 10° was chosen for this analysis. The recording procedures were accurate to only 2–5°, and even beetles in control situations had standard deviations of 8–15° in their turn angles (Akers and Wood, 1989a). Any much smaller bin size seemed inappropriate. The results for both the 30- and 60-min diffusion periods were extremely similar. Only the results from the 60-min period experiment are shown for illustration.

The beetles' movements were very irregular or noisy (Figure 7 in Akers and Wood, 1989a). Some orientation mechanisms might be expected to produce noisy tracks, while others would more likely produce more regular oscillatory or patterned movements. Therefore an analysis of the variation of the “overall turn” or “arc” sizes of the swings on a beetle's track was made. A “turn”

could mean the turn angle at a particular point on a beetle's track. However, a track is more usually perceived, not as a series of points, but as a series of "arcs" or "swings" or "turns." An arc is usually recognized when a series of turn angles all have the same sign, or at least mostly so, and their signs are opposite to those of the series of turn angles just preceding and following the arc in question. To avoid confusion, the use of "turn" will be avoided, and "turn angle" and "arc" will be used in the above sense.

A program was developed to analyze the number of degrees turned in an arc. It was based upon the following set of criteria, which was intended to filter out variation due to recording procedures and very small turn angles made by the beetles: (1) Each point was considered sequentially and, if its turn angle was of the same sign as the immediately preceding points, it was summed with those points. (2) If the turn angle was of the opposite sign from the preceding points, several different possibilities were evaluated. (2a) If its absolute angle was greater than 10° , it was accepted as initiating a new arc. Ten degrees was chosen because it was about twice the measuring error for a single turn angle (Akers, 1985; Akers and Wood, 1989a) but still less than half the average turn angle even for control beetles. (2b) If the turn angle was less than 10° , the next point on the path was checked. (3a) If the sum of the points was more than 15° and if both points were of the same sign, they were accepted as a new arc. (3b) If a turn angle was not accepted as a new arc, it was summed into the previous arc.

The above algorithm filtered out the very noisy arcs, and its determinations of arcs were generally in accordance with subjective judgments in the sense that, where the program indicated an arc, an arc would be recognized. The standard deviation of the arc sizes within each track was used to probe for regular movements. There was no a priori reason to suppose that every beetle should have a similar mean arc size, i.e., the size of arcs made by a beetle might well have a strong component due to the individual beetle. However, if regular oscillatory or patterned movements were occurring, the arcs of a given beetle should all be of a similar size, and the variation of the arc size should be low for every beetle, regardless of each beetle's particular mean arc size. A consideration of the general heading of the beetle during an arc was also important. Several of the mechanisms might conceivably produce a track that would oscillate regularly back and forth about a heading of 0° . However, only in those in which the basic turn pattern was centrally generated should the regular, oscillatory movements continue regardless of the heading with respect to the source. Mechanisms based on a balance of sensory input ought not generate regular, uniform turns at headings away from 0° . Accordingly, the program also calculated the net heading of each arc. Forty-five degrees was chosen as an arbitrary cutoff for arcs with a heading near 0° , for the purpose of pooling the data.

The maximum absolute heading with respect to the source was determined

between occurrences where the heading was 0° . This measured how far away a beetle turned from the source before turning back towards it, and thus gave a measure of the heading at which a beetle initiated its turns back towards the source. If an insect turned beyond 180° or went in a circle, this heading was defined as 180° .

The effects of distance from the source on gross heading were explored graphically and by linear regressions. Similar analyses were done for walking and turning rates (Akers, 1985), but are not shown here. Turn (=arc) radii were not measured directly but were estimated from walking and gross turning rates using the relationship:

$$r_c = (360 \times WR)/(2 \times \pi \times GTR),$$

where r_c = turn radius, WR = walking rate, and GTR = gross turning rate.

The relationship between the gross (absolute) heading of a beetle at a point and the gross turn angle that was subsequently made at that point was estimated by linear regression, to reveal any tendency of the beetle to stabilize on a heading of 0° . Similar regressions were made between the net (actual) heading of a beetle at a point and the net turn angle that was subsequently made at that point (Bell and Tobin, 1981, 1982), to reveal any mechanism where the insect was able to more or less efficiently estimate the direction towards the source.

RESULTS

Frequency histograms of turn angle size showed that the distribution was centered about 0° and that it fell off smoothly and without any intermediate peaks at higher turn angles (Figure 1). The distributions were unimodal, indicating that the beetles apparently did not have two distinct turn angle sizes within the track, such as might occur if tight arcs were interspersed with relatively straight sections.

No consistent pattern in the analysis of overall arc size appeared among the dosages (Table 1), except that the beetles responding to a source consistently turned through more degrees of arc than beetles in blank arenas. The overall coefficients of variation were in the range of 60–90% (calculated from Table 1). This does not seem to be a very low amount of variation, since it would produce an error confidence interval for the arc size at least as large as the mean itself. Coefficients of variation for moths zigzagging in an odor plume appear to be 20–30% (Kuenen and Baker, 1983; Cardé et al., 1984). By contrast, the beetles' movements are highly irregular (examples of trails may be found in Figure 7 of Akers and Wood, 1989a). The variation was similar both when the heading was less than and greater than 45° , although the variation was possibly a bit smaller at headings less than 45° . Low variation at both low

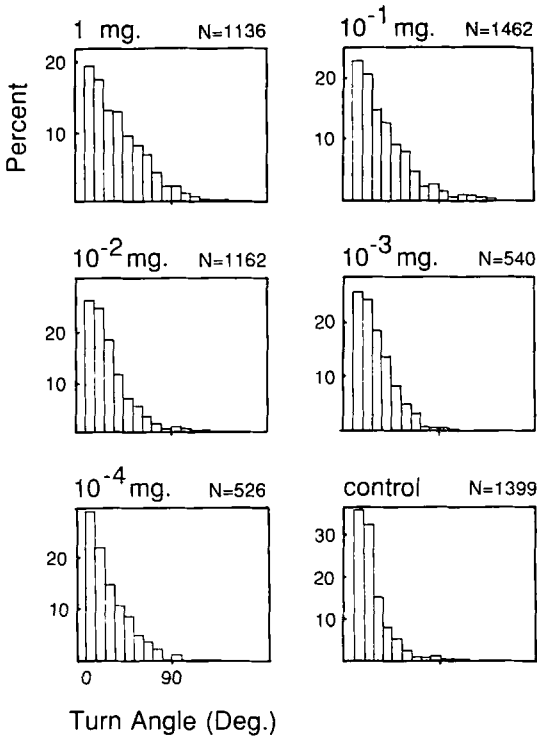


FIG. 1. The number of occurrences of turn angle sizes within each 10° interval in each treatment of the experiment with the 60-min diffusion period. The unimodal distribution suggests there were not two classes of turn angles sizes, such as might have been expected if straight sections were interspersed with bouts of turning. N = total number of turn angles in treatment.

and high headings would have been consistent with regular movements driven by a central pattern generator, while low variation at low headings and high variation at headings farther from the source would have been consistent with regular movements driven by a highly efficient mechanism based on sensory input. High variation at both low and high headings would seem more consistent with mechanisms where the movements are derived from chemosensory input.

In comparing the gross (absolute) heading of a beetle at a point to the absolute turn angle that was subsequently made at that point, the most important result was that the intercepts of the regression lines were always significant and in the range of $20\text{--}30^\circ$ (Table 2). Thus, the beetles continued to have moderate rates of turning even when they were headed almost directly towards the source.

TABLE 1. EFFECTS OF DOSAGE AND HEADING OF ARCS ON ARC SIZE

Diffusion period (min)	All arcs			Mean net headings > 45°		Mean net headings < 45°	
	Dosage (mg)	Mean of mean arc sizes (deg)	Std. dev. of arc size within tracks (deg)	Mean of mean arc sizes (deg)	Std. dev. of arc size within tracks (deg)	Mean of mean arc sizes (deg)	Std. dev. of arc size within tracks (deg)
30	1	90.4	62.1	82.5	57.4	91.2	53.6
	10 ⁻¹	96.8	60.2	106.4	56.2	97.0	49.7
	10 ⁻²	79.4	64.6	72.4	55.6	79.7	49.6
	10 ⁻³	86.6	63.6	83.1	49.8	93.3	64.9
	10 ⁻⁴	96.0	73.7	93.7	63.5	89.6	66.9
	control	62.8	55.1	60.5	40.1	80.8	53.7
Mean, not including control			87.6 ± 12.9	56.5 ± 4.9	90.1 ± 6.5	56.9 ± 8.4	
60	1	96.9	66.8	101.7	61.2	91.0	54.8
	10 ⁻¹	86.5	58.7	93.4	57.2	80.2	52.5
	10 ⁻²	74.4	66.6	80.7	65.3	64.8	48.0
	10 ⁻³	85.0	65.8	98.2	52.0	78.3	56.4
	10 ⁻⁴	98.7	60.4	91.5	67.5	92.6	49.2
	control	53.5	51.2	51.0	46.1	73.8	47.5
Mean, not including control			93.1 ± 8.0	60.6 ± 6.2	81.4 ± 11.2	52.2 ± 3.6	

TABLE 2. REGRESSIONS OF ABSOLUTE TURN ANGLE AT EACH DATA POINT VS. ABSOLUTE HEADING

Dosage (mg)	Diffusion period (min)	Number of turns	Intercept (degs)	P^a	Slope (degs/deg)	P	Correlation coefficient (r)
1.0	30	1289	30.5	<0.0001	0.075	<0.0001	0.119
10^{-1}	30	1308	26.2	<0.0001	0.092	<0.0001	0.160
10^{-2}	30	790	27.6	<0.0001	0.044	0.033	0.067
10^{-3}	30	487	25.5	<0.0001	0.086	0.0005	0.151
10^{-4}	30	729	25.5	<0.0001	0.038	0.040	0.066
1.0	60	1136	29.0	<0.0001	0.116	<0.0001	0.173
10^{-1}	60	1462	26.9	<0.0001	0.076	<0.0001	0.132
10^{-2}	60	1162	22.8	<0.0001	0.044	0.002	0.087
10^{-3}	60	540	24.1	<0.0001	0.011	0.516	0.033
10^{-4}	60	526	25.0	<0.0001	0.039	0.078	0.063

^aProbability of observing a T statistic larger than the observed T given that H_0 is true. H_0 : value of parameter = 0.

The slopes of the regressions were often significant and always positive, although they were low (Table 2). Thus, the beetles tended to have slightly larger turn angles at higher headings.

In the analysis of the net (actual) heading of a beetle at a point as compared to the net turn angle that was subsequently made at that point, the regressions showed the slopes were highly significant (Table 3), but they were in the range of -0.1 to -0.2 , and the correlation coefficients were in the range of -0.15 to -0.30 . The sign of a turn angle therefore had a weak tendency to be opposite to the sign of the heading at that point.

Frequency histograms of the maximum absolute heading between occurrences of headings of 0° suggested that the distributions on the full circle were centered about 0° and fell off gradually towards the higher angles, with a small peak at 180° (Figures 2 and 3). There was no consistent sign of any peak at intermediate angles, although there was a suggestion of peaks at 50 – 70° and 90 – 110° . Most of the maximum headings were less than 90° . However, headings between 90 and 180° were not uncommon. Calculations from the regressions of maximum heading versus distance to the source further showed that, on average, turns back towards the source were initiated at about 70° , even at the release point of the beetles (Table 4).

The heading decreased gradually as the source was neared, becoming steeper within 1 – 5 cm of the source (Figures 4 and 5). Turn radius decreased as the source was neared (Figure 6) (Akers, 1985).

TABLE 3. REGRESSIONS OF ACTUAL TURN ANGLE AT EACH DATA POINT VS. ACTUAL HEADING

Dosage (mg)	Diffusion period (min)	Number of turns	Intercept (degs)	P^a	Slope (degs/deg)	P	Correlation coefficient (r)
1.0	30	1289	-2.00	0.097	-0.159	<0.0001	-0.261
10^{-1}	30	1308	0.05	0.962	-0.116	<0.0001	-0.218
10^{-2}	30	790	-1.20	0.396	-0.115	<0.0001	-0.218
10^{-3}	30	487	-2.92	0.091	-0.104	<0.0001	-0.188
10^{-4}	30	729	-2.36	0.082	-0.072	<0.0001	-0.157
1.0	60	1136	0.10	0.939	-0.203	<0.0001	-0.308
10^{-1}	60	1462	1.05	0.328	-0.105	<0.0001	-0.198
10^{-2}	60	1162	3.16	0.001	-0.065	<0.0001	-0.149
10^{-3}	60	540	4.82	0.0003	-0.093	<0.0001	-0.223
10^{-4}	60	526	1.22	0.449	-0.078	<0.0001	-0.171

^aProbability of observing a T statistic larger than the observed T given that H_0 is true. H_0 : value of parameter = 0.

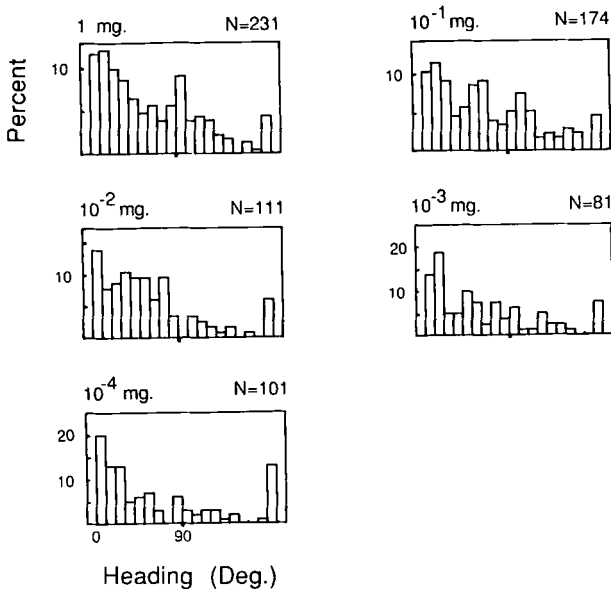


FIG. 2. The number of occurrences of maximum headings within each 10° interval after having turned from a heading of 0° and before returning to it. Data are from each treatment in the experiment with the 30-min diffusion period. N = total number of maximum headings in treatment.

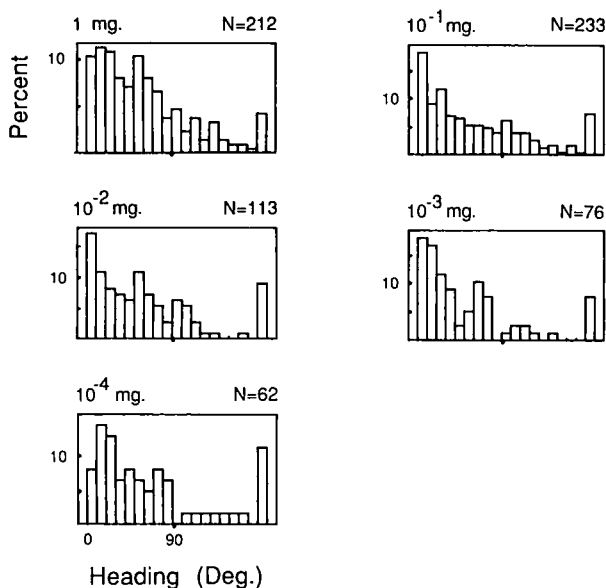


FIG. 3. The number of occurrences of maximum headings within each 10° interval after having turned from a heading of 0° and before returning to it. Data are from each treatment in the experiment with the 60-min diffusion period. N = total number of maximum headings in treatment.

TABLE 4. REGRESSIONS OF MAXIMUM HEADING ON EACH ARC VS. DISTANCE FROM SOURCE

Dosage (mg)	Diffusion period (min)	Number of arcs	Intercept (degs)	P^a	Slope (degs/cm)	P	Correlation coefficient (r)
1.0	30	231	42.8	<0.0001	1.94	<0.0001	0.267
10^{-1}	30	174	32.8	<0.0001	2.98	<0.0001	0.447
10^{-2}	30	111	54.8	<0.0001	0.31	0.656	0.085
10^{-3}	30	81	37.7	0.002	2.30	0.024	0.226
10^{-4}	30	101	31.4	0.007	2.65	0.002	0.285
1.0	60	212	45.2	<0.0001	1.77	0.002	0.200
10^{-1}	60	233	32.2	<0.0001	2.66	<0.0001	0.364
10^{-2}	60	113	67.9	<0.0001	-0.67	0.392	0.048
10^{-3}	60	76	34.7	0.080	1.47	0.002	0.167
10^{-4}	60	62	44.4	0.002	2.07	0.061	0.204

^aProbability of observing a T statistic larger than the observed T given that H_0 is true. H_0 : value of parameter = 0.

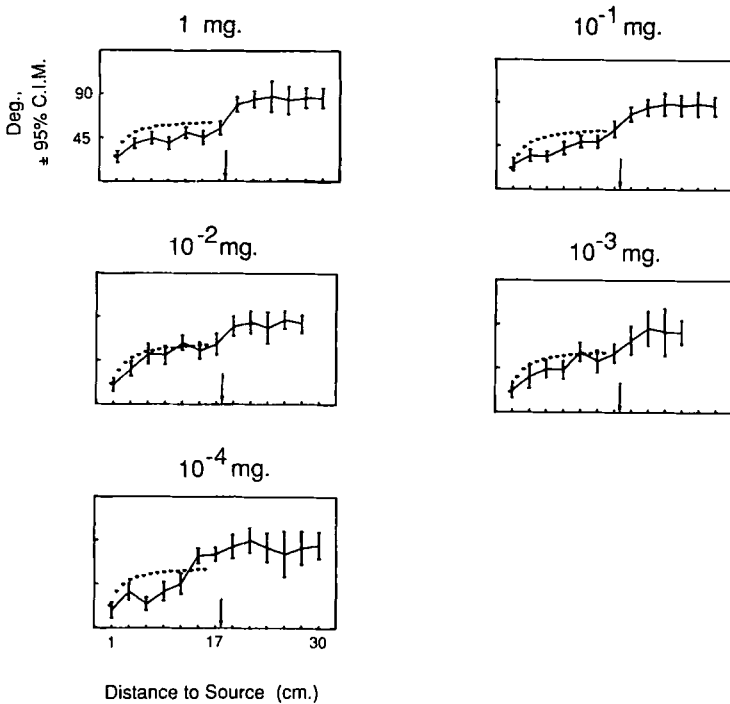


FIG. 4. The effect of distance from the source on the mean gross heading in the experiment with the 30-min diffusion period. Error bars show the 95% confidence interval of the mean. Dotted lines show headings predicted by a model based on counterturns initiated by a decrease in the rate of increase of concentration. Arrow marks the approximate release point of the beetles.

DISCUSSION

Since Fraenkel and Gunn (1961) published their benchmark work on orientation, the many mechanisms that are potentially available to an animal for orientation have gradually become more evident (e.g., Mittelstadt-Burger, 1972; Jander, 1975; Kennedy, 1977a,b, 1978, 1983, 1986; Bell and Tobin, 1982). The mechanisms the beetles used to orient to a point source in still air were inferred from a set of hypotheses about the characteristics of track form produced by each of several possible mechanisms. Measurements were found or devised to describe different characteristics of track form, and then the observed measurements were compared to those that were expected from each of the mechanisms. However, while analyses such as the present one may be strongly

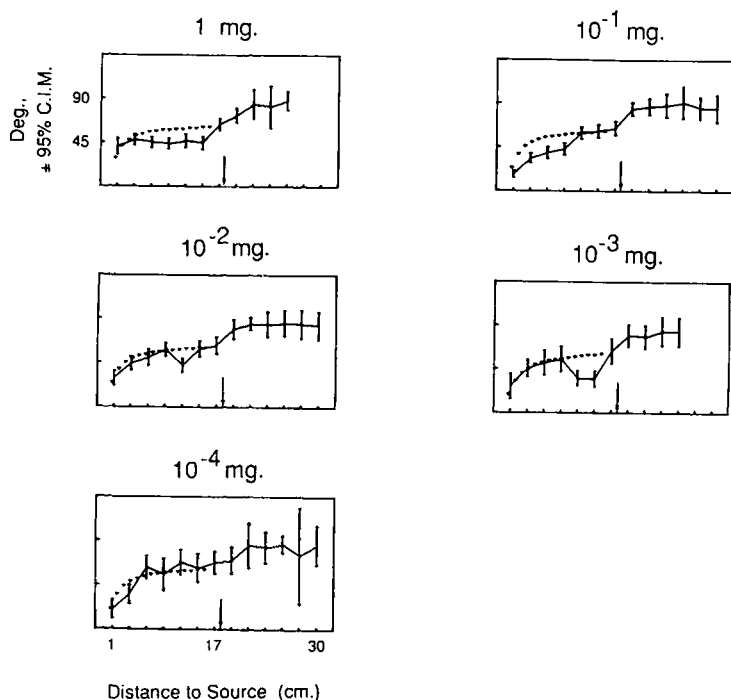


FIG. 5. The effect of distance from the source on the mean gross heading in the experiment with the 60-min diffusion period. Error bars show the 95% confidence interval of the mean. Dotted lines show headings predicted by a model based on counterturns initiated by a decrease in the rate of increase of concentration. Arrow marks the approximate release point of the beetles.

suggestive, they cannot be completely conclusive without further experiments that isolate individual mechanisms (Bell and Tobin, 1982; Kennedy, 1977b). Because of the considerable state of flux in the theory and terminology of orientation, each mechanism will be explicitly defined as it is considered. They will be discussed using the terminology of Fraenkel and Gunn (1961), as extended by Kennedy (1986) and herein. Although Kennedy developed many of his ideas while working with anemotactic situations, with some care they can be extended to a purely chemotactic situation, as will be attempted here.

The counterturning schemakinesis best explained the observed results if the cue used to initiate the counterturns was a decrease in the rate of increase of concentration. It was further necessary to postulate that the beetles have some innate tendency to compensate for the size of a previous arc in the opposite direction (Mittlestadt-Burger, 1972). In a schemakinesis, an animal uses infor-

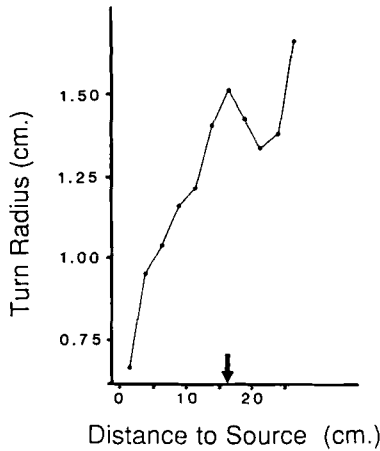


FIG. 6. An example of the relationship between distance from the source and the mean turn radius, as calculated from the walking and gross turning rates within 2.54-cm increments of radius from the source. Data are from the 1-mg dosage with a 60-min diffusion period. Arrow marks the approximate release point of the beetles.

mation concerning the behavior of stimulus intensity through time, combined with proprioceptive or central information concerning its own movements, to guide its path to the source (Kennedy, 1986). This is unlike many of the more classic mechanisms, where the animal is guided towards the source primarily by information on its position or orientation relative to the geometry of an intensity gradient (Fraenkel and Gunn, 1961). In the counterturning schemakinesis, an animal turns in one direction until it senses a critical sensory cue, at which time it initiates a turn in the opposite direction (Kennedy, 1986).

In a purely tactic situation, the environmental cue used to initiate a counterturn will strongly affect the form of the track. For example, consider an animal moving in a radial gradient, wherein contour lines of equal concentration form a series of concentric circles. If an animal depends upon a decrease in intensity to initiate a counterturn, each arc will have to obtain a heading beyond 90° before the animal can encounter such a decrease, because the animal does not move away from the source at headings less than 90° . On the other hand, an animal could use the rate of increase of intensity to initiate counterturns before a heading of 90° . As heading decreases towards 0° , the rate of increase of concentration increases, while as heading increases away from 0° , the rate decreases. The rate of increase of intensity begins to decrease when a heading of 0° is crossed.

Counterturning is consistent with the unimodal distribution of turn angle sizes (Figure 1). Turning is essentially constant in this mechanism, and there

is nothing to cause two or more classes of turn angle size, such as might occur if straight sections were interspersed with tight arcs.

The beetles' movements were irregular and noisy, as indicated by the analysis of arc size (Table 1) (examples of trails are shown in Figure 7 of Akers and Wood, 1989a). Counterturning is consistent with such noise, because the counterturns are initiated largely or entirely by sensory input and therefore are liable to the full effects of sensory noise.

The comparison of absolute heading at a point with the absolute turn angle made at that point indicated that turning rate remained moderate even at headings near 0° (Table 2), meaning that the beetles had no tendency to fix on a heading directly towards the source. This is entirely consistent with counterturning. However, the beetles also tended to have slightly larger turn angles at higher headings. There is no clear reason why counterturning alone ought to produce larger turn angles at larger headings. However, there are two possible contributing factors. First, the beetles may have some innate tendency to initiate arcs in the direction opposite to the current arc, and this tendency may grow stronger the longer it takes to be released by sensory cues. There is some support for this suggestion. Beetles in control situations do not have gross turning rates low enough to account for their low net turning rates (Akers, 1985; Akers and Wood, 1989a). In other words, control beetles have much straighter paths overall than would be the case if all their turn angles were of one sign. Therefore, the beetles probably have some innate tendency to compensate for the size of a previous arc. Mittelstadt-Burger's work also supports the possibility of an innate counterturning tendency that compensates for the size of a previous arc (Burger, 1971; Mittelstadt-Burger, 1972), and Kramer (1975) found that moths walking in pheromone-free wind periodically reverse their heading with respect to the wind in a manner that produces fairly direct movement overall. The second possible mechanism that could produce larger turn angles at higher headings is a change in the cue used to initiate a counterturn as heading changed, from a decrease in the rate of increase of concentration to a decrease in concentration itself. However, while exploratory graphs showed turn angle increasing as heading increased, no pattern was consistent enough to allow a decision on this second possibility.

Compensated counterturning is consistent with the low slope and correlation coefficient observed in the comparison of the net (actual) heading of a beetle at a point with the net turn angle that was subsequently made at that point (Table 3). In counterturning, turn angles would be away from as well as towards a heading of 0° , which would decrease the correlation, but if there were compensation for the size of a previous arc, the slope would be negative.

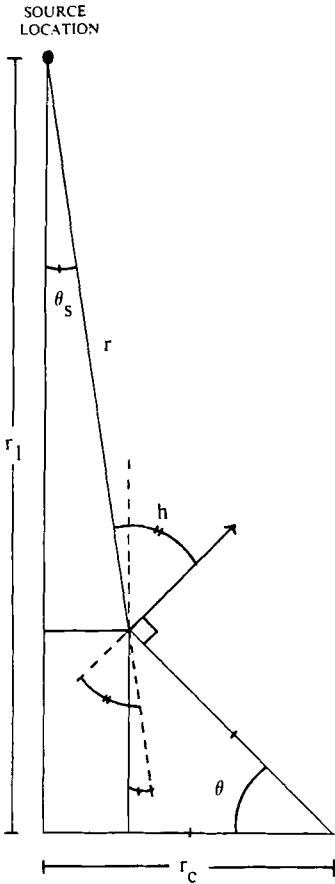
The analysis of the maximum absolute heading between occurrences of headings of 0° (Figures 2 and 3) gave the first indication that, if counterturning were in operation, a cue for counterturn initiation was likely to be a decrease

in the rate of increase of concentration. Most maximum headings occurred before 90° , the heading beyond which a decrease in concentration becomes possible. These counterturns, then, could not have been initiated by a decrease in concentration. Calculations from the regressions of maximum heading versus distance to the source further showed that, even at the release point of the beetles, counterturns were initiated at about 70° , on average (Table 4), consistent with the possible minor peak in maximum headings at $50\text{--}70^\circ$. In addition, counterturning could produce a distribution centered near 0° , if the cue used to initiate counterturns were a decrease in the rate of increase of concentration, because the rate of increase begins to decrease just as a heading of 0° is crossed. Finally, counterturning has no difficulty explaining those maximum headings that occurred between 90 and 180° , because these counterturns could have easily been initiated by a decrease in concentration. A decrease in concentration would begin to occur just beyond 90° , where the second possible minor peak in maximum heading also appears to lie.

Counterturning was also best able to explain the form of the decrease in heading as the source was neared (Figures 4 and 5), but only if the cue used to initiate counterturns was a decrease in the rate of increase of concentration. If a counterturn were initiated by a decrease in concentration, heading would not decrease much, because, at any distance, the animal still must head away from the source, i.e., at a heading of greater than 90° , to encounter a decrease in concentration. If the animal used a decrease in the rate of increase to trigger a counterturn, we need to know how concentration changes with heading as the animal turns, in order to predict how heading would decrease as the source is neared. If we assume that the beetles move along roughly circular arcs, then the rate of change of distance to the source with respect to heading can be determined (Figure 7). The relationship between concentration and distance to the source may then be substituted into the equations. This latter relationship was estimated from gas-liquid chromatography data, under the assumption that the gradient is circular (Akers and Wood, 1989a).

In order to better visualize the behavior of the system, the equations were evaluated at 1° intervals over arcs of 0° to 90° , using a computer to carry out the calculations. If turn radius is held constant, the rate of increase decreases more rapidly with heading the closer an arc is to the source, but decreasing turn radius can strongly offset this effect (Akers, 1985). The effect of changing turn radius can be taken into account by calculating the turn radius from regressions relating the walking and turning rates to distance from the source (see Methods and Materials). For the purposes of exploration (Figure 8), the regression parameters used to estimate the turn radii were the means over all treatments except the control.

The rate of increase is always maximal at a heading of 0° , directly towards the source, and falls to 0 at 90° , where the animal is moving neither away from



$$r^2 = (r_1 - r_c \sin \theta)^2 + (r_c - r_c \cos \theta)^2$$

$$r = (r_1^2 - 2r_c r_1 \sin \theta - 2r_c^2 + 2r_c^2)^{\frac{1}{2}}$$

$$\frac{dr}{d\theta} = \frac{r_c^2 \sin \theta - r_c r_1 \cos \theta}{r}$$

$$h = (90 - (90 - \theta)) + \theta_s$$

$$= \theta + \theta_s$$

$$= \theta + \arctan \left[\frac{r_c - r_c \cos \theta}{r_1 - r_c \sin \theta} \right]$$

$$\frac{dh}{d\theta} = \frac{r_1^2 - r_c r_1 \sin \theta - r_c^2 \cos \theta + r_c^2}{r^2}$$

Where:

- r = distance from beetle to source
- r₁ = distance at which arc was initiated (heading = zero degs.)
- r_c = turn radius of arc
- h = heading angle of beetle
- θ = angle turned by beetle along arc

The relationship between heading and motion along the arc is a one-to-one mapping, at least when r_c < r₁ and h < 90 deg. Therefore the derivative of the function relating arc angle to heading is the inverse of the derivative of the function relating heading to arc angle. that is,

$$\frac{d\theta}{dh} = \frac{1}{\frac{dh}{d\theta}}$$

Then, by the chain rule,

$$\frac{dr}{dh} = \frac{dr}{d\theta} \frac{d\theta}{dh} = \frac{dr}{d\theta} \frac{1}{\frac{dh}{d\theta}}$$

FIG. 7. The geometry and equations needed to analyze the rate of change of distance from the source with respect to heading. The animal is assumed to turn along circular arcs of turn radius r_c.

or towards the source (Figure 8). Any arbitrarily chosen amount of decrease from the maximum is still reached at considerably lower headings as the source is neared, despite the accounting for the decrease in turn radius. For example, if the animal were sensitive to an absolute decrease of 0.01 units, heading would be expected to decrease in a strongly linear manner as the source was neared (Figure 9). The linear relationship and especially the steepness of the decrease

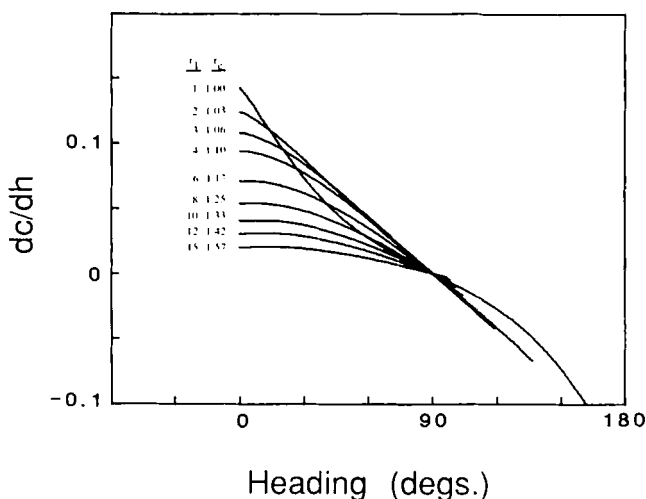


FIG. 8. An example of the effects of heading, turn radius, and distance of arc initiation from the source of pheromone on the rate of change of concentration with respect to heading, under the assumptions of a radial gradient and circular arcs. The y axis units are arbitrary and derive from a gradient decreasing exponentially with distance from the source and with concentration at the source arbitrarily set to one. r_1 and r_c are defined in Figure 7. The r_c for each r_1 was calculated from regressions relating the walking and gross turning rates to distance from the source. The parameters used in the regression equations were the means of the parameters over all treatments but the control.

is different from that seen in the data (Figures 4 and 5). On the other hand, if the animal were, for example, sensitive to a 50% decrease from the maximum rate of increase, heading would decrease gradually farther from the source, becoming steeper as the source was neared (Figure 9).

If the animal indeed initiated a counterturn when it just perceived a change in the stimulus, counterturn initiation would occur at the "just noticeable difference" of psychophysics, and the behavior of the headings might be expected to follow the classic Weber's and Fechner's laws. These laws state that the difference in stimulus intensity needed to obtain a just noticeable difference in sensation magnitude is proportional to the original stimulus intensity (Gescheider, 1976). Therefore, a model based on a proportionate decrease is also physiologically reasonable.

The dotted lines in Figures 4 and 5 show the predicted changes in heading as the source is neared, for a counterturning schemakinesis when the cue is a 50% decrease in the rate of increase. The turn radii were calculated using the respective parameters for the regressions of walking and turning rate in each

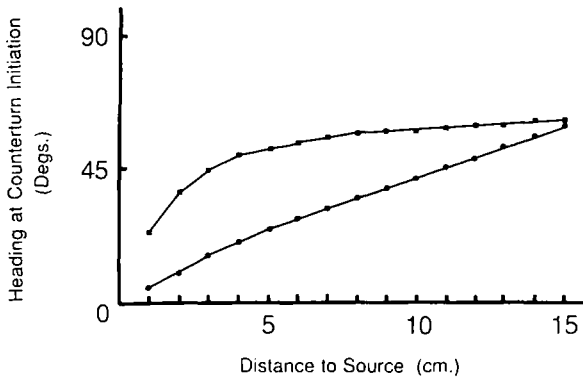


FIG. 9. The predicted headings at counterturn initiation derived from Figure 8 and under the assumption that counterturns are initiated by either an absolute decrease of 0.01 units from the maximal rate of increase (lower curve, circles) or a proportional decrease of 50% from the maximal rate of increase of concentration (upper curve, squares). Each point on a curve is derived by finding the curve in Figure 8 that has the same distance from the source as that point, then moving down from the maximum rate of increase on that curve until the chosen critical decrease from the maximum is reached. The heading at which that decrease was reached is then read from the horizontal axis.

treatment. While somewhat arbitrary, a 50% decrease was chosen because it produced critical headings of about 60° at the starting distance of 15 cm from the source. Values of $45\text{--}65^\circ$ are typical for the mean gross headings at that distance. Although the analysis required several simplifying assumptions, the predicted curves often approximate the actual data in form, if not actually lying on the line (Figures 4 and 5). This is particularly true in the 60-min experiment (Figure 5). Perhaps a slight difference in the shape of the gradient in the 30-min experiment was responsible for the slightly steeper decreases in heading observed in that experiment (Figure 4). The relationship between concentration and distance from the source was estimated using data from the 60-min diffusion period (Akers and Wood, 1989a), while no information is available on the exact form of the gradient in the 30-min experiment.

A similar process of comparing expected and observed results was applied to each of the other possible mechanisms of orientation. Each appeared to be inconsistent with at least some observations. The classic tropotaxis is inconsistent with several of the results. In the tropotaxis, symmetrically placed receptor organs permit simultaneous comparisons of intensity in space. If the organism turns towards the receptor with the greater intensity, it will turn towards the source. If it can then balance the stimuli efficiently on its receptors, its path to the source should be very direct (Fraenkel and Gunn, 1961).

The paths of the beetles towards the source were generally far from direct (Figure 7 in Akers and Wood, 1989a), and the beetles were turning almost continuously. The comparison of the gross (absolute) heading of a beetle at a point with the gross turn angle that was subsequently made at that point (Table 2) showed that the beetles continued to have moderate rates of turning even when they were headed almost directly towards the source. Thus, the beetles never appeared to fix on a heading near 0° . In addition, in the regressions of the net (actual) heading of a beetle at a point on the net turn angle that was subsequently made at that point (Table 3), the slopes were in the range of -0.1 to -0.2 , and the correlation coefficients were in the range of -0.15 to -0.30 . If the animal was perfectly tropotactic, and given the conventions by which the signs of angles were defined, the regressions should have had a slope of -1.0 and a correlation coefficient of -1.0 (Bell and Tobin, 1981). These observations, then, are inconsistent with an efficient tropotaxis. However, an inefficient tropotaxis, where the animal cannot accurately balance the intensity on the two receptors, is still a possibility. Such a tropotaxis is consistent with the above results, with a unimodal distribution of turn angle sizes (Figure 1), and with the increase in turn angle size as heading increased away from 0° (Tables 2 and 3).

Nevertheless, a tropotaxis cannot explain those maximum headings of 90° to 180° that occurred between occurrences of headings of 0° (Figures 2 and 3). The difference between the antennae in distance to the source reaches a maximum at a heading of 90° and then begins to decrease again as the animal turns away from the source. If a tropotactic animal does not perceive the difference in concentration by the time it achieves a heading of 90° , that difference will once again begin to decrease and make it even less likely that the animal will initiate a return towards the source. It would more likely continue on until it completes a circle. The possible minor peak in maximum heading, which appears to occur at about 90 – 110° , could be attributed to tropotaxis, but the argument would seem more satisfactory if the peak were centered just before 90° rather than beyond it.

Neither is the tropotaxis consistent with the form of the decrease in heading as the source was neared or with the decrease in turn radius. As distance from the source decreases, smaller separations in space should be needed to obtain the same amount of information concerning the gradient. In a tropotaxis, the difference in intensity between two receptors separated by some fixed distance will increase, thereby improving its possible efficiency. Accordingly, heading should decrease. However, since the animal should then head more linearly to the source as the source is neared (Fraenkel and Gunn, 1961), turn radius should increase. This is opposite to the behavior observed (Figure 6) (Akers, 1985).

Furthermore, although an inefficient tropotaxis might be expected to cause a decrease in heading as the source was neared, the expected form of the decrease

does not fit the data. An analysis similar to that done for the counterturn was done for the tropotaxis (Figure 10) and predictions made as to the heading at which the animal would encounter a concentration difference large enough between its antennae to cause it to turn back towards the source after having once turned away (Figure 11). Again, for Figure 11, the critical concentration difference was taken to be that which occurred at a heading of 60° and a distance of turn initiation of 15 cm, and the parameters of the regressions for the walking and turning rates were the means of the parameters of all treatments but the control. The receptors were treated as point receptors in this analysis, but this is a reasonable estimate given the beetle's club-shaped antenna.

Two points become apparent from this analysis. First, neither curve fits the observed data (Figures 4 and 5). Second, the difference in concentrations between antennae at a distance of 15 cm and a heading of 60° is on the order of 3%. The animal must be able to detect this difference if the tropotaxis is to

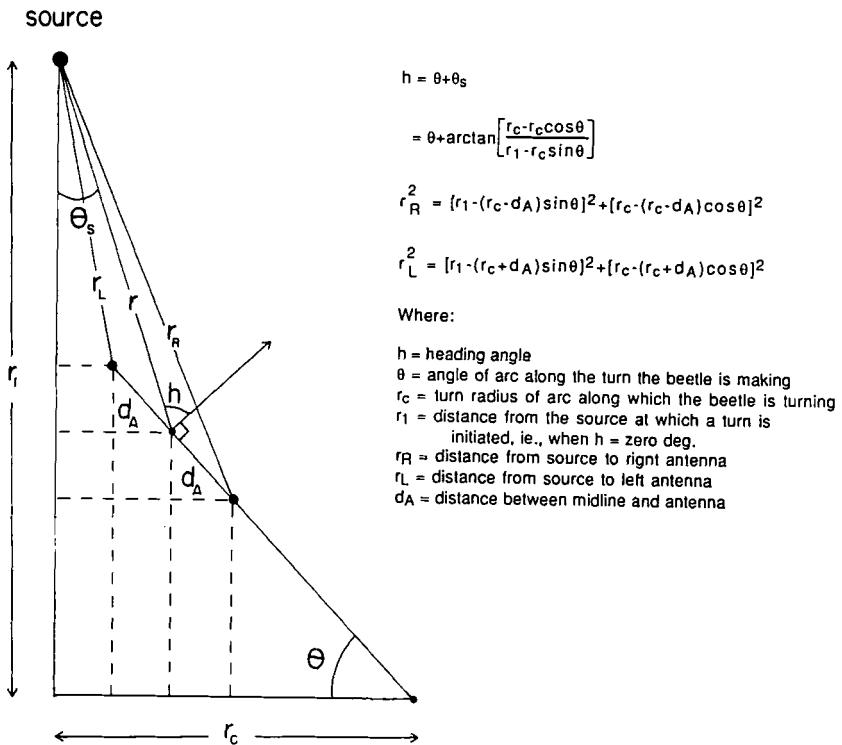


FIG. 10. The geometry and equations needed to analyze the difference in distance to the source between two point receptors placed symmetrically about an animal's midline. The animal is assumed to turn along circular arcs.

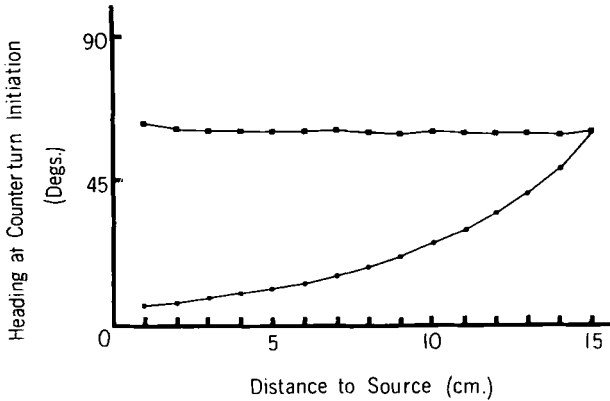


FIG. 11. The headings at which an animal would turn back towards the source after having once turned away, as predicted by an inefficient tropotaxis which requires either a critical absolute (lower curve, circles) or proportional (upper curve, squares) difference in concentration between the antennae before a return towards 0° can occur.

function. However, this implies a Weber fraction of 0.03, considerably lower than the values of 0.3–1.5 that have been obtained for other animals and modalities (Gescheider 1976). The few direct measurements that have been made on insects indicate that the ability to detect a 3% difference may be an order of magnitude better than any performance previously observed (Martin, 1964; Kramer, 1975; Bell and Tobin, 1982). On the other hand, the 50% decrease for counterturn initiation is within the usual range for Weber fractions.

Both the classic klinotaxis of Fraenkel and Gunn (1961) and a closely related modification of it are also inconsistent with several of the behavioral observations. In the classic klinotaxis, short “wigwags” of the receptor organs are made to either side of the body and information on the stimulus intensity in space is obtained by comparing stimulus intensity through time. The wigwags are herein assumed to be generated within the central nervous system. Oriented maneuvers are made on the basis of information obtained about the symmetry of the gradient during the wigwags. However, unless the wigwags are large enough that the concentrations can be made different enough at the ends of the swings, a classic klinotactic movement will not gain the animal much information regarding the form of the gradient (Kennedy, 1986).

A small modification to the classic klinotaxis overcomes this problem. Since it would generate a pattern that appears analogous to the zigzagging behavior of flying moths (Kennedy, 1983), it shall be referred to as the zigzagging mechanism. The animal still obtains information concerning the symmetry of the gradient as it executes centrally generated arcs, but the two mechanisms

differ in scale. In the classic klinotaxis, the arcs are executed by swinging the anterior portion of the body back and forth, and the size of each wigwag is smaller than the body length of the animal, or certainly very little more. In the modified klinotaxis, the arcs may be many body lengths of the animal and are executed by a regular alteration of the walking pattern. Straight sections between arcs could also occur.

The small wigwag movements that would be expected under a klinotaxis are inconsistent with the size of movements made by the beetles. The beetles are 4–6.5 mm long, and their turn radii were typically in the range 0.7–1.5 cm (Figure 6). The circumferences of the arcs made by the beetles therefore averaged two to five body lengths, clearly much larger than would be expected of the klinotaxis. However, such arcs would easily be consistent with zigzagging. Zigzagging is also consistent with the unimodal distribution of turn angle sizes, if no straight sections occurred between arcs (Figure 1). In addition, zigzagging is consistent with a moderate rate of turning even at headings close to 0° , although there is no clear reason why zigzagging should produce larger turn angles as heading increased, or why these turn angles should compensate in sign for the heading (Tables 2 and 3).

Further, zigzagging is inconsistent with the noise apparent in the movements of the beetles (Figure 7 in Akers and Wood, 1989a). This was also indicated by the analysis of the variation of arc sizes (Table 1), which failed to demonstrate the low level of variation that might be expected from regular, oscillatory movements. The turns in zigzagging are herein postulated to be driven largely by a central pattern generator, and such movements tend to be regular, especially once they have come into balance with environmental requirements (review in Camhi, 1984). Examples of such movements may be found in leg movements in walking, wing movements in flight, swimmeret movements, leech swimming movements, and respiratory movements. Even the zigzagging of moths in a windstream may be driven by a central pattern generator (Kennedy, 1983, 1986), although there is now some renewed controversy on this point (Preiss and Kramer, 1986). Coefficients of variation for moths zigzagging in an odor plume appear to be 20–30% (Kuenen and Baker, 1983; Cardé et al., 1984). By contrast, the beetles' movements are highly irregular, with coefficients of variation in the range of 60–90% (calculated from Table 1). Since variation was always fairly high, rigid zigzagging seems unlikely.

Zigzagging was also inconsistent with the distribution of maximum headings between occurrences of 0° , which was centered about a heading very close to 0° (Figures 2 and 3). Zigzagging might have been expected to produce maximum headings centered some distance from 0° , as the animal turned on its regular, centrally defined arcs. For instance, track headings of the straight sections in zigzagging moth flight are generally in the range of 30–90° with respect

to the wind (Kuenen and Baker, 1983; Cardé et al., 1984; Kennedy et al., 1980; Farkas et al., 1974).

Zigzagging was also inconsistent with the notable decrease in heading as the source was neared (Figures 4 and 5). Since the turns in zigzagging are postulated to be under central control, heading should not change much under this mechanism, despite possible changes in sensory stimulation. However, the path might become narrower as the source was neared, which would be reflected in a decrease in turn radius (Akers, 1985).

Two other possible orientation mechanisms became apparent during the course of this analysis, but they also appeared to be inconsistent with the results. The first is a modification of the zigzagging mechanism and was termed the "look and leap." In zigzagging, oriented movements have to interact with instructions for movements arising from the basic turn-pattern generator, or else the animal would probably never reach a source. In the look and leap, the animal separates movements to gather information from oriented movements, in order to minimize the amount of turning. It first initiates an orientation movement, based on an endogenously generated pattern of turns, during which it obtains information about the symmetry or form of the gradient. Based upon this sensory information and information concerning the form of its own movements, the animal estimates the direction towards the source, and aligns itself with it. It then continues in a more or less linear fashion until an endogenous or sensory cue triggers another orientation maneuver. Apparently, some flies use a similar mechanism to orient with respect to the wind in response to an odor cue (the "aim first, then shoot" mechanism, Bursell, 1984; Hawkes and Coaker, 1979; Miller and Strickler, 1984; Kennedy, 1986).

The second mechanism is termed the "steepest-ascent schemakinesis." If an organism could efficiently measure the rate of change of the stimulus, it might attempt to identify that heading with the steepest rate of increase in intensity, which would be directly towards the source. The path might be expected to consist of relatively straight segments, during which the animal would determine the rate of increase along that heading, punctuated by sharp turns.

In both the above mechanisms, linear sections of track are interspersed with bouts of rapid turning. They might then be expected to generate a bimodal distribution of turn angle sizes (Figure 1), which was not observed. However, the sharp turns in the steepest ascent might be relatively rare, and thus perhaps obscured in a distribution. Furthermore, both mechanisms could conceivably operate without the appearance of a bimodal distribution of turn angles or turn radii. If an animal had relatively regular turn angles at every point on its track, it could create relatively linear sections by alternating the signs of the turn angles and larger arcs by holding the sign constant for a period of time. I have not yet conceived a simple measurement that would reflect such a behavior. Such

behavior is not obvious in samples of tracks (Figure 7 in Akers and Wood, 1989a) and it seems difficult to reconcile with the variation in turn angle size (Figure 1). Nonetheless, the possibility cannot be completely excluded. Otherwise, the steepest ascent schemakinesis is inconsistent with the nearly constant turning executed by the beetles, and the beetles never seemed to head very directly towards the source, which also might have been expected. In the look-and-leap mechanism, the animal is also postulated to fix on a heading towards the source, once it estimates its direction from the orientation maneuver. Accordingly, it should not turn much for a period while on a heading near 0° , while at higher headings its turn angles should generally be considerably larger. However, the comparison of the absolute headings and turn angles at a point on the track showed that the beetles continued to have moderate rates of turning even when they were headed almost directly towards the source (Table 2). Combined with the lack of any regular movements and the unimodal turn angle size distribution, the beetles apparently did not look and leap very well.

In summary, the numerical analysis of the behavioral responses indicated that a compensated counterturning schemakinesis was most consistent with the observations, if the sensory cue used to initiate a counterturn was a decrease in the rate of increase of concentration. However, this raises the question of whether a beetle could detect such a cue at all. Concentration itself increases only about 30% as the animal swings from a heading of -90° to 90° , or vice versa. The detection of this increase itself would imply a Weber fraction of 0.3, relatively low by classic psychophysics. Detection of a rate of increase would require comparing at least two successive increases within this interval. On the other hand, Kramer (1978) has indicated that moths can detect a decrease of as little as 1%/sec, if continued for 5 sec. If this is true of the beetles as well, then they could possibly make the comparisons necessary for the mechanism to function. Finally, the possible effects of habituation have not been addressed in this paper. Because the animal is moving in a gradient, the stimulus is constantly changing, and thus any habituation is very difficult to isolate in the present experiment. However, in a preliminary experiment on the purely kinetic effects of pheromone (Akers, 1985), pheromone was applied as uniformly as possible to the arena, in an attempt to minimize any gradients. In that experiment, walking and turning rates were very similar between early (0–10 sec) and late (45–55 sec) portions of the track, giving at least some indication that pheromone perception habituates only slowly in this species, if at all.

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BEHAVIORAL RESPONSE OF SOLITARY FATHEAD MINNOWS, *Pimephales promelas*, TO ALARM SUBSTANCE

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Abstract—Single fathead minnows, *Pimephales promelas*, were exposed to a range of concentrations of conspecific skin extract. Their responses were observed qualitatively and quantified by a computer linked to an activity meter. The response of fathead minnows to skin extract is complex, involving at least three separate types of behavior. The minnows responded over a 1000-fold range of extract concentrations with combinations of dashing, freezing, slowing, and exploring. The latency of the response increased at the lowest extract concentrations, suggesting summation of sensory cell responses. At low stimulus concentrations, a period of exploratory behavior sometimes preceded the more typical alarm responses. The active space generated by the alarm substance in 1 cm² of minnow skin may exceed 58,000 liters.

Key Words—Schreckstoff, alarm pheromone, fathead minnow, *Pimephales promelas*, fish behavior, club cells, alarm substance cells.

INTRODUCTION

Alarm substance (Schreckstoff) is released, by mechanical damage, from specialized alarm substance cells (ASCs) in the skin of ostariophysan fishes and initiates a defensive response, or "fright reaction," in the receiver (reviews: Pfeiffer, 1977; Smith, 1986). As with most fish pheromones, the exact chemical nature of the alarm substance is not known. There is developing evidence that hypoxanthine-3(*N*)-oxide is an important component of the alarm signal (Pfeiffer, 1982; Pfeiffer et al., 1985), but a single compound may not account for the species specificity that has been reported in the ostariophysan alarm system (e.g., Schutz, 1956). The response of the receiving fish has been reported to

vary among species in ways that seem generally appropriate to the habits of the species (Pfeiffer, 1977). Pfeiffer et al. (1986) examined the fright reaction of 15 species and found a generalized reaction consisting of rapid swimming (possibly controlled by Mauthner cell discharge), followed by goal-directed avoidance or shelter-seeking behavior.

Research on the effects of acidic pH on the response of fathead minnows to alarm substance (Smith and Lawrence, 1988) required description of the fright reaction as measured by an automatic recording system (Lemly and Smith, 1986). This description and the experiments conducted to find appropriate stimulus levels for the pH experiment provided new information on the characteristics of the fright reaction. A combination of visual observation, using video equipment, and sensitive measurement of times and distances, using a computer-based activity meter, allowed us to distinguish several components in the response of the fish to alarm substance. The constraints of the system limited the study to lone fish confined in shallow water.

In nature, the response of prey animals to predators may vary with the degree of risk or vulnerability. In general, risk and distance from the predator will be inversely related. In a chemical warning system dilution will often indicate distance. We might expect that prey will respond as if predators are close at high alarm substance concentrations, and as if predators are more distant at greater dilutions of alarm substance. Similarly there may be responses that are performed as a first response and others that are more frequently the second or third step in the fright reaction, and the appropriate first response may vary with distance and hence dilution.

Active space, the volume in which concentration of the stimulus exceeds the threshold of the receiver, is a key attribute in a chemical warning system. Active space is presumably an adaptive trait based on selection of sender and receiver for a combination of stimulus concentration and sensory threshold that provides adequate warning without too many unnecessary responses. The findings of this study allow the calculation of a conservative value for the active space, per surface area of skin, of fathead minnow alarm substance.

METHODS AND MATERIALS

Experimental Animals and General Handling Procedures. The majority of the fathead minnows were collected at Zelma Reservoir, approximately 70 km southeast of Saskatoon, Saskatchewan, Canada. Other fish were from Eagle Creek, approximately 100 km southwest of Saskatoon. The fish were held in temperature-controlled outdoor pools until they were used between April and August 1986. The experimental fish were moved from the outdoor pools to glass aquaria (45–60 liters) where they were sorted by size. Fish 5-cm or more in

fork length were retained and held for approximately two weeks at 16°C under a 12:12 light-dark photoperiod. They were fed daily with Tetramin, a commercial fish food.

After this initial adjustment period, the fish were transferred to the experimental room in groups of 10–15 and held up to a week in 45-liter glass aquaria. From this holding facility, fish were transferred in pairs to a series of Plexiglas acclimating tanks and finally to the testing tank (Lemly and Smith, 1985, 1986, 1987). The fish were acclimated and tested under incandescent lighting of 55 lux as measured at the water surface, on a 12:12 light-dark cycle. Testing occurred between 0830 and 1030 hr at a mean water temperature of 23°C.

To ensure that experimental fish would display relatively "undisturbed" behavior, a series of two acclimating tanks was used prior to placing the fish in the testing tank. Acclimating and testing aquaria were identical in size, water quality, and the provision of a centrally moored floating shelter. In the first acclimating tank the water depth was 8–10 cm and two fish were present. After 24 hr both fish were transferred to a second acclimating tank with water depth of 4–5 cm, the same depth as in the testing aquarium. After 24 hr the individual fish that seemed least disturbed were selected as the experimental fish and transferred into the testing tanks for the final 24 hr. The other fish was discarded. This procedure was necessary to allow the fish to adjust to being alone in shallow water. The fish were fed after each transfer.

Testing aquaria, tubing, and filters were drained, thoroughly rinsed, and refilled immediately after each trial to ensure the longest possible adjustment time to the testing tank. The washing and water exchange also minimized exposure of experimental fish to alarm substance from the previous trial. After each trial the experimental fish were measured, weighed, and sexed. For the dilution experiment the overall sex ratio was 23 females (mean wt. 2.16 g) to 47 males (mean wt. 2.69 g).

Activity Tracking System. The system consisted of an Opto-Varimex-Aqua tracking meter (Columbus Instruments) that surrounds a Plexiglas aquarium. This system has been described in detail by Lemly and Smith (1986). Recessed inlet and outlet manifolds on the aquarium were attached to one another by Tygon tubing that ran through a power filter and back into the test aquarium. The activity tracking meter (ATM) was interfaced to a microcomputer that integrated the digitized signal from the grid of light beams in the meter and tabulated data on position and activity of a single fish for a designated time period. At regular intervals, the computer "scans" the light beams for breaks. A minimum time between beam scans of 0.125 sec was chosen after pilot testing showed that this interval was short enough to record accurately the movement of a rapidly swimming fish. Of the eight behavior parameters measured by the meters, six proved useful in assessing fathead minnow alarm responses: total distance traveled, time spent resting, number of stereotypic movements, time

spent in stereotypic activity, time spent in ambulatory movement, and total number of beam breaks. Stereotypic activity is a term used by Columbus Instruments to describe activity in which the fish breaks light beams without moving out of a "box" of beams of preset dimensions, one grid square. Ambulatory movement refers to movement in which the fish progresses steadily from one region of the tank to another. The total number of beam breaks per unit time provided an index of general activity. The variables measured by the ATM are not totally independent nor are they all directly correlated. Together they allow the experimenter to characterize the response of a particular species to a stimulus.

In this study each trial consisted of eight identical time periods of 2.13 min. The alarm substance stimulus was injected into the circulation system at the end of the fourth time period. A loop of the circulation system, between the power filter and the inlet manifold, passed into an adjacent room allowing a hidden observer to inject the stimulus through the tubing. Video equipment allowed the remote observer to see and record the behavioral responses of the fish as well as to simultaneously monitor the quantitative data from the ATM.

Alarm Substance Preparation. Fifteen donor fish were selected, weighed, measured (fork length), and sexed immediately after capture from an outdoor pool. Mature males that might have seasonally reduced numbers of ASCs (Smith, 1973) were not used. The sex ratio of the donor fish was nine females to six males. The mean weight was 1.5 g and the mean length was 5.1 cm. A patch of skin was removed from each side of the caudal peduncle area of each fish. A small subsample was preserved in 10% formalin for histological examination and determination of ASC abundance. These samples were embedded in paraffin and 7- μ m sections were stained with periodic acid-Schiff's reagent and counterstained with hematoxylin. ASCs were counted and measured in representative sections. The relative area of ASCs and epidermis were determined using an image analyzing computer. ASCs were present in 14 of the 15 donors. A damaged sample prevented assessment of the 15th fish. The approximate total area of minnow skin used to prepare the alarm substance was 11.9 cm². This skin was homogenized in 100 ml of glass-distilled water (GDW) using a Polytron homogenizer and then filtered to remove scales and tissue. A portion of the stock solution was immediately frozen (-18°C) in 5-ml quantities, another portion was diluted 1 part stock to 4 parts GDW and frozen in 5-ml portions. After a period of initial testing, four different dilutions were chosen for more intensive testing: (1) stock solution, (2) a 1:10 dilution of the stock, (3) a 1:100 dilution, and (4) a 1:1000 dilution. With the exception of the stock solution that was used as previously prepared, the solutions were made by slowly thawing (the test tube was set in a beaker of cold water and allowed to thaw at room temperature) the original 1:4 dilution, adding appropriate amounts of GDW, and refreezing in 5-ml portions. Immediately prior to use in the exper-

imental apparatus, the prepared alarm substance was slowly thawed, as above, and 1 ml was drawn into a syringe. Fifteen fish were tested at each concentration. Solutions were tested in random order to minimize effects of seasonal changes in behavior and of possible contamination. Control injections of 1 ml of GDW were also tested on 15 fish.

Statistical Analyses. Data from the three time periods immediately preceding the alarm substance injection were pooled and compared with pooled data from the three time periods immediately following the stimulus using Wilcoxon's matched-pairs, signed-ranks test (Sokol and Rohlf, 1981). Data were analyzed separately for each of the six behavioral parameters for each stimulus dilution. Differences were considered to be significant at probabilities less than 0.05.

RESULTS

Description of Alarm Behavior. Video observations indicated that the most common response to the alarm substance stimulus was to become motionless, freezing, for periods ranging from approximately 0.5 min to greater than 8 min (the entire poststimulus time). The next most frequent alarm behavior was slowing, in which the fish would slow its rate of movement, e.g., less circling and nibbling under the shelter and fewer, shorter forays away from the shelter. Nibbling and circling under a shelter are common types of fathead behavior (McMillan and Smith, 1974). Dashing, the least common response, was actually the first part of a biphasic response. Several seconds to approximately 1.5 min of very rapid, apparently disoriented swimming were followed most often by the fish becoming motionless, freezing, or by the fish showing a very low level of movement, slowing. A nonalarm behavior, exploring, typified by slow, open-water swimming towards and along the inlet channel, was observed when alarm substance stimulus levels of <0.01 were injected. Exploratory swimming lasted from slightly under a minute to just over 2 min. In six of seven cases an obvious alarm reaction, either freezing (4/6) or slowing (2/6), was observed to follow the initial exploratory behavior. A portion of the experimental population did not respond to the alarm substance stimulus; human qualitative assessment indicated 21% nonresponders while machine assessment indicated 13% (Smith and Lawrence, 1988).

Effects of Stimulus Dilution on Alarm Behavior. The majority of fathead minnows were still capable of sensing and reacting to the lowest dilution of alarm stimulus tested (Table 1 and Smith and Lawrence, 1988). Exploring was observed only at the two lowest stimulus concentrations (Figure 1) and caused an initial increase in distance traveled in the first poststimulus interval in the 0.001 treatment group (Figure 2). A comparison of the last pre-

TABLE 1. THE EFFECTS OF DILUTIONS OF STOCK SOLUTION ON THE BEHAVIORAL RESPONSE OF FISH TO INTRODUCTION OF SKIN EXTRACT^a

Parameters	Before/ after	Dilutions				
		Stock	0.1	0.01	0.001	Control
Distance	B	1008	870	1463	1099	869
	A	280	327	682	669	739 ^{ns}
Time resting	B	185	142	187	196	214
	A	291	229	276	249	211 ^{ns}
Stereotypic time	B	170	212	161	157	148
	A	84	141	90	11	161 ^{ns}
Ambulatory time	B	30	29	35	30	22
	A	9	11	18	19 ^{ns}	20 ^{ns}
Stereotypic moves	B	298	336	287	251	235
	A	127	209	170	194	248 ^{ns}
Beam breaks	B	972	1124	954	993	842
	A	449	602	564	595	806 ^{ns}

^aB = before stimulus introduction. A = after stimulus introduction. Distance was measured in centimeters, time in seconds, and moves and beam breaks were counted. Values are the mean scores of 15 fish over the three intervals (total 6.39 min) preceding stimulus introduction and the three intervals following stimulus introduction. All differences are significant ($P < 0.05$), except those marked "ns," based on a Wilcoxon matched-pairs, signed-ranks test comparing each individual fish's cumulative score before the stimulus with that fish's score after the stimulus.

stimulus interval with the first poststimulus interval indicated that both the stock and 0.1 stimuli initiated significant decreases in distance traveled ($P < 0.05$, Wilcoxon matched-pairs, signed-ranks test), but the difference between these two intervals in the 0.01 and 0.001 stimuli was not significant. The frequency of freezing as the first response was positively correlated with stimulus strength ($P = 0.05$, Spearman rank correlation coefficient; Siegel, 1956). However, freezing often followed exploring so the total frequency of freezing declined only slightly. There was an observable increase in the latency of the response as stimulus concentration was reduced. With stock and 0.1 dilutions, the response was initiated almost immediately after injection, whereas at 0.001 the alarm response was delayed (Figure 2). This delay could be demonstrated by comparing stereotypic activity for the last prestimulus and first poststimulus intervals. The two intervals differed significantly in the three highest dilutions ($P < 0.01$, Wilcoxon matched-pairs, signed-ranks test) but at the 0.001 dilution the difference was not significant ($P > 0.05$). In the 0.001 dilution the second

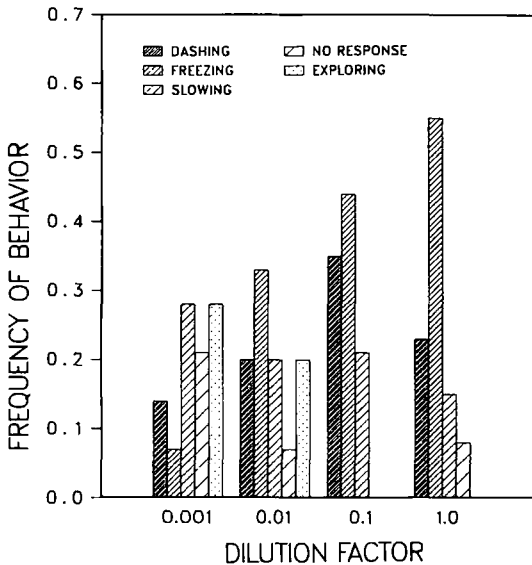


FIG. 1. Proportional frequencies, as a first response, of behaviors in the post stimulus intervals after introduction of skin extract at four dilutions of the stock solution.

poststimulus interval was significantly different from the last prestimulus interval ($P < 0.05$).

Since we know the area of skin used in preparing the stock solution and the various dilutions performed, we can estimate the active space of alarm substance per cm^2 . The original stock solution contained 11.9 cm^2 of skin diluted in 100 ml of water. That stock solution diluted to 1 : 1000 still produced a significant reaction when 1 ml was added to an ATM containing 7 liters of water. This indicates that 1 cm^2 of fathead minnow skin contains enough alarm substance to reach threshold levels in 58,823.5 liters, equivalent to a cube approximately 3.9 m on a side. Since the 1 : 1000 dilution was still a very effective stimulus, the real active space may considerably exceed our estimate. The initial exploring response occurred in the 0.01 dilution but not in the 0.1 dilution, indicating that the active space at which the transition from an immediate fright reaction to an initial exploration occurs presumably lies between 588 liters/cm^2 and 5882 liters/cm^2 . Our measurements of epidermal thickness (mean = 0.052 mm), cell diameter (mean 0.025 mm), and proportion of epidermis composed of ASCs (11.7%) permit a rough calculation of the amount of ASC material in 1 cm^2 of skin in our sample ($6.1 \times 10^{-3} \text{ mm}^3$) and of the active space generated per average ASC (80 liters). A conservative conclusion, considering the high

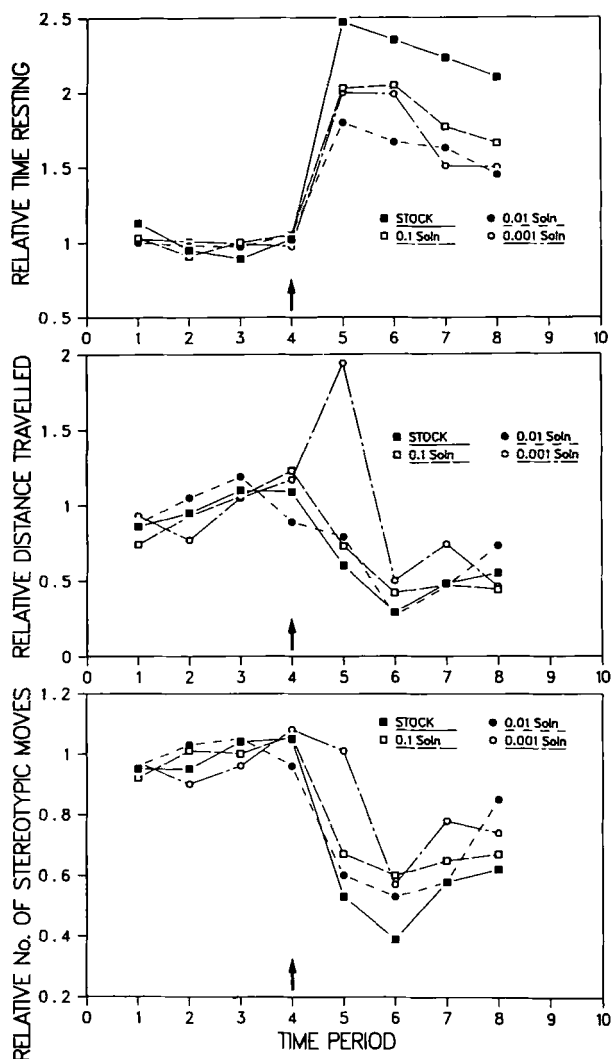


FIG. 2. Changes in relative levels of three behavior patterns over time, before and after introduction of skin extract at four dilutions of the stock solution. The stimulus was introduced at the end of the fourth time interval (2.13-min intervals). Average activity of each test fish over the first four intervals has been indicated on the Y axis as 1.0. Arrows indicate alarm substance introduction.

variation among samples and uncertainties such as the proportion of ASCs broken by the tissue disruption process, would be that the contents of a single ASC could generate an active space of the order of 10s of liters.

DISCUSSION

Lone fathead minnows exposed to skin extracts performed four general types of behavior: dashing, reduced activity (slowing and freezing), exploring, and no response. The use of lone fish was necessitated by the limitations of the activity tracking meter. Being alone may be a relatively rare situation for minnows in nature, and the behavioral responses may have been modified by the stress of being alone. Although they are schooling fish, there are some situations in which fathead minnows are alone in nature. Male fatheads are often alone on their territories during the spawning season (Unger, 1983), and females have been observed moving singly into the territories during the spawning season (Smith, personal observation). Males retain their response to alarm substance during their territorial phase (Smith, 1976). Using lone fish had the advantage of yielding a "pure" response to the chemical stimulus. Schooling minnows have been reported to show a fright reaction to the sight of other fish responding (Verheijen, 1956). Any response with more than one fish present may be influenced by visual as well as chemical stimuli.

The dashing component of the multiphase response to alarm substance may be an escape response controlled by the Mauthner neurons, as suggested by Pfeiffer et al. (1986). The behavior we observed was consistent with his descriptions of Mauthner responses in other fishes. Sudden unpredictable dashing may be appropriate as an initial response to imminent danger. The Mauthner response has been hypothesized to be escape behavior (Eaton and Bombardieri, 1978). It can be initiated by mechanical or visual stimuli as well as by alarm substance. Freezing and slowing may follow dashing or may occur as initial responses without a preceding dashing phase. It is not clear whether freezing and slowing are two different responses or different intensities of the same response. Both may make the minnow less conspicuous but freezing is probably more effective in this regard. Similar reductions in activity are components of the fright reaction in other cyprinids such as the pearl dace, *Semotilus margarita*, where they are associated with physiological indicators of stress (Rehnberg et al., 1987).

The exploring behavior that occurred at low stimulus concentrations may be a response to low alarm substance concentrations or to other chemical components of the skin extract. The behavior is generally similar to the response of fathead minnows to feeding stimuli (Lemly and Smith, 1985, 1987) and the skin extract will contain amino acids and other compounds that may elicit food searching. Bardach and Todd (1970) suggested that alarm substance may be attractive in low concentrations but provided no evidence or arguments to sup-

port this idea. Our results leave the question unresolved. The answer may have to await the availability of chemically pure alarm substance. Increased response latency at low stimulus concentration is typical of stimulus summation (Getchell, 1986). In fathead minnows increased response latency was often associated with exploring behavior, which may bring the minnow into contact with more alarm substance, speeding the summation process. Exploring may be similar to the "predator inspection behavior" described by Magurran (1986) in the European minnow, *Phoxinus phoxinus*, behavior thought to provide the prey with information about the predator.

The active space of a semiochemical depends on attributes of both the sender and the receiver. The sender determines the concentration of the semiochemical through the amounts produced and released. In the minnow alarm substance system this is presumably adjusted through control of the density of ASCs in the skin. In fathead minnows ASC density is known to be under endocrine control during the breeding season. Males lose their ASCs during the breeding season in response to increased androgen levels and regain them later as androgen declines (Smith, 1973, 1974). The factors controlling ASC numbers in females and nonbreeding fish are not known. The receiver determines the threshold of the response, either through sensory capabilities or central controlling mechanisms. Virtually nothing is known about the sensitivity of fathead minnows to chemical stimuli. The data reported here suggest that they detect and respond to low concentrations of skin extract. Calculations based on the work of Gandolfi et al. (1968) suggest an active space of $> 10,000$ liters/cm² of skin in the zebra danio, *Brachydanio rerio*, the same order of magnitude as our findings. Such calculations of active space provide an opportunity to compare laboratory findings with subjective impressions of the usefulness of the alarm substance in the wild. Active spaces with dimensions of several meters, containing 10s of thousands of liters seem to be within the range that should be useful to a 5-cm fish. There are a number of uncertainties in applying these findings to natural situations, including lack of knowledge of the amount of skin damage caused by natural predation or of the spatial distribution of alarm substance plumes in natural waters.

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FLIGHT RESPONSE OF *Heliothis subflexa* (GN.) FEMALES (LEPIDOPTERA: NOCTUIDAE) TO AN ATTRACTANT FROM GROUNDCHERRY, *Physalis* *angulata* L.¹

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Abstract—Mated female *Heliothis subflexa* (Gn.) (HS) moths 1–7 days old responded positively in a Plexiglas flight tunnel to an attractant extracted with methanol from fresh whole-leaf washes of groundcherry, *Physalis angulata* L. Response to the groundcherry extract, as indicated by plume-tracking (i.e., upwind flight toward the odor source) and contact with the chemical dispenser did not change significantly during the first 5 hr of scotophase. Overall, ca. 50% of the responding moths also landed on the chemical dispenser; ca. 50% of the moths that landed also deposited eggs. There were no significant differences in the behavioral responses of females mating only once and those that had mated two or more times. Virgin females and male moths were significantly less responsive to the groundcherry attractant than mated females. The flight tunnel bioassay described provides an excellent system for evaluating plant allelochemicals associated with host-plant selection.

Key Words—*Heliothis subflexa*, Lepidoptera, Noctuidae, attractant, kairomone, groundcherry, *Physalis angulata*, flight tunnel bioassay, plant–insect interaction.

INTRODUCTION

Environmentally controlled systems are needed to study host-finding behaviors of phytophagous insect species. The present study was designed to develop a

¹This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by USDA.

system that could be used to evaluate host-plant attractants and associated behaviors (e.g., oviposition) in a controlled environment. *Heliothis subflexa* (Gn.) (HS) was selected as the test subject because of its limited host range (*Physalis* spp.) (Brazzel et al., 1953) and also because of potential changes in plant selection behaviors that might occur as a result of hybridization between HS and its sibling species *H. virescens* (F.) (HV) (Laster, 1972). Little is known about the nature of flight responses of *Heliothis* moths to host-plant chemical stimuli.

METHODS AND MATERIALS

A Plexiglass flight tunnel was used to observe the behavior of HS to crude plant extract (Mitchell and Heath, 1987) obtained by washing fresh, whole groundcherry leaves in methanol. Typically, 100 g of groundcherry leaves were extracted for 30 sec in 500 ml of methanol. The extract was filtered and concentrated to 1 g/ml with a rotary evaporator. The 60 × 60 × 185-cm tunnel was located in an environmentally controlled room 3 × 2.6 × 2.1 m. The room was maintained at ca. 26.8°C with a heat pump/air conditioner and ca. 50% relative humidity with a room humidifier. Overhead fluorescent lights (two banks of two 40-W bulbs) connected to an electric timer were used to control the reversed light-dark cycle (14 hr light; 10 hr dark). Three 25-W red light bulbs were spaced equally above the tunnel and remained on continuously. Air was pulled through the tunnel at 0.5 m/sec and was exhausted via a 30-cm-diam. flexible pipe with a fan. An intake vent located in the wall of the environmental chamber allowed a continuous flow of fresh air into the room from the outside.

The HS used in the tests were obtained from our laboratory colony, which was started from ca. 300 larvae collected from groundcherry in the fall of 1983 and has had new genetic material introduced each fall since 1984 (Mitchell et al., unpublished data). The larvae were reared on a modified pinto bean diet (Guy et al., 1985). The insects were sexed in the pupal stage and separated before emergence of adults. Upon emergence, the adults were confined either in 3.8-liter cages as virgins or combined for mating (21 females and 14 males/cage) and held until the desired age for testing. Insects were held under the same environmental conditions as were maintained in the flight tunnel room. On the day of testing, the moths were sexed by gently squeezing the abdomen to extrude the genitalia and the females were placed in a 25 × 25 × 50-cm Plexiglas holding cage, which was placed in the flight tunnel room ca. 1 hr before scotophase.

Each test was begun by introducing 1-g equivalent of groundcherry-methanol extract into the tunnel on the evaporator substrate (white muslin) placed over the end of a glass cylinder dispenser with a 3.5-cm opening. Air was blown

through the cylinder and cloth into the tunnel with an aquarium pump at a rate of 1 liter/min to provide a continuous flow (plume) of the extract odor through the tunnel. The location of the plume through the center of the tunnel was verified previously by observing a smoke trail, produced by introducing smoke into the dispenser system. Each moth was removed individually from the holding cage and placed into a cylindrical 4×6.5 -cm plastic release cage with screened ends. After placement of the release cage into the downwind end of the flight tunnel, the moth was released immediately and observed for 2 min. Behavioral responses were timed and recorded. All female moths were dissected after testing to confirm mating status. Tests with untreated controls were conducted periodically to ensure that response to the dispenser substrate (white muslin) was negligible when treated with solvent (methanol) only and that contamination of the bioassay system had not occurred. Data were analyzed by ANOVA or the unpaired *t* test (Steel and Torrie, 1960).

Experiment 1. Females 1, 2, 3, 4, 5, 6, or 7 days old, which had been confined with males for mating, were tested to determine the effects of moth age on response to the groundcherry extract. Tests were conducted 1–4 hr after the beginning of scotophase. Specific responses, including flight initiation, type of flight (either undirected or plume tracking, i.e., following the plume trail upwind), contact with or without landing on the extract-treated substrate (white muslin), and duration (in sec) of each response were recorded. Three replications of 10 individuals each were completed for each age group.

Experiment 2. A second experiment was conducted to determine if response to the extract changed significantly as the scotophase progressed. Mated female moths 3–6 days old were tested in the flight tunnel as described for the first experiment. The tests began ca. 10 min after the onset of scotophase and continued through the 5th hr of darkness. Data were tabulated from two replications of 37 individuals each for each hour.

Experiment 3. In the third experiment, 3- to 5-day-old mated HS female moths were tested for their response to groundcherry extract in the flight tunnel as described previously. Special attention was focused on detecting any previously unobserved differences in the behavior of insects in the different response groups; i.e., (1) those that flew at random (undirected flight), (2) those that responded by tracking the plume trail from the extract dispenser, (3) those that made very brief contact with the extract-treated substrate but did not land, and (4) those that actually landed on the dispenser substrate. The time (sec) that each moth in each response group remained in the release cage before taking flight, the time spent in undirected flight, the number and duration (sec) of plume trackings, and when contacts and landings occurred also were recorded. Data collected from 3- to 5-day-old moths in experiments 1 and 2 were combined with the experiment 3 data to provide three replications of 150–170 individuals each.

Since all of the HS females used in the various experiments were dissected to determine mating status, the data were tabulated to determine if the number of matings per female had any effect on moth response to the attractant. The number of matings per female was determined by the number of spermatophores counted in each moth upon dissection.

Also, in experiment 3, the response of virgin HS females and males (0, 2, or 4 days old) to the groundcherry extract was determined during the first 3 hr of scotophase (three replications) using the criteria and methods described previously.

RESULTS AND DISCUSSION

Mated female HS moths in all age groups (experiment 1) demonstrated a positive anemotactic response to groundcherry extract (Figure 1). There were no significant differences in total response (59–71%) among the age groups (1–7 days old) (Figure 2). An average of 71% (1 day old), and 79% (7 days old) to 100% (4 days old) of the moths that followed the attractant plume also made contact or landed. Of these, 20% (7 days old) and 25% (1 day old) to 55% (4 days old) and 61% (3 days old) landed on the treated surface. There was a significant correlation ($P < 0.05$, $r = 0.821$, 5 *df*) between moth age and the percentage of moths that contacted the groundcherry extract without landing on the treated substrate. Seven-day-old moths made the most contacts (46%) but had the fewest landings (13%) (Figure 2).

There were no significant differences among the age groups in the mean number of contacts (1–3) per female before landing on the extract-treated substrate or in the number of repeated contacts (1.5–4.8, including initial contact) when making contact without landing (Table 1). Also, moth age had no significant effect on (1) mean number of times a moth flew up the plume before landing, (2) number of times that an individual flew up the plume and made contact without landing, or (3) the number of plume trackings (1–2) by moths that responded without contacting the dispensing substrate (Table 1).

Response to groundcherry extract by mated HS female moths (experiment 2) did not change significantly from 10 min to 5 hr after the initiation of darkness. The percent that either tracked the plume, contacted, or landed on the extract-treated substrate ranged from 84% during the first hour and 75–77% from hours 2–4, to 58% for those flown 4–5 hr after dark.

In experiment 3, 73% of the mated female HS moths responded to groundcherry extract by flying up the plume, contacting the dispenser, or landing (Table 2). There were no significant differences in the seconds before flight initiation (8.3–13.2) among the moths that did not respond to the extract and those that demonstrated a positive response. Although the resting position was sometimes

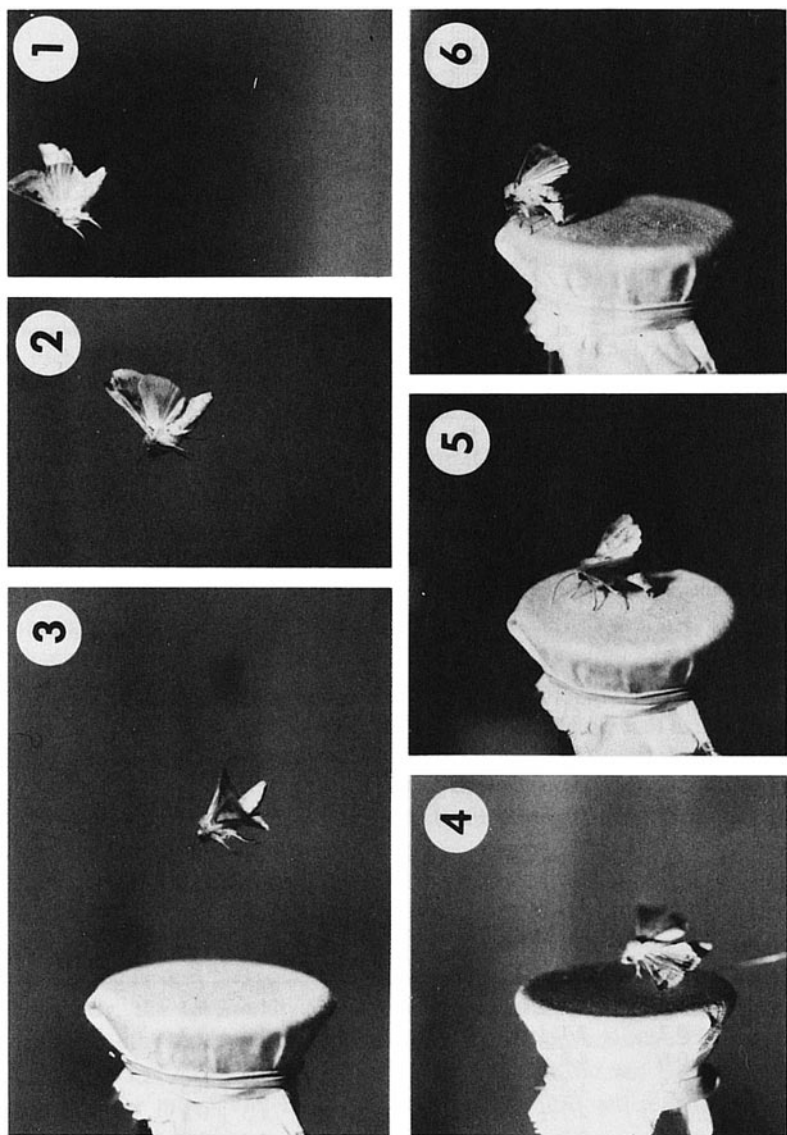


FIG. 1. Flight sequence of *Heliothis subflexa* in a positive anemotactic response to 1-g equivalent of methanol extract from whole-leaf washes of fresh groundcherry leaves in methanol. Response includes plume tracking (1-3), contact with the extract-treated substrate (4), and landing (5-6) with possible oviposition.

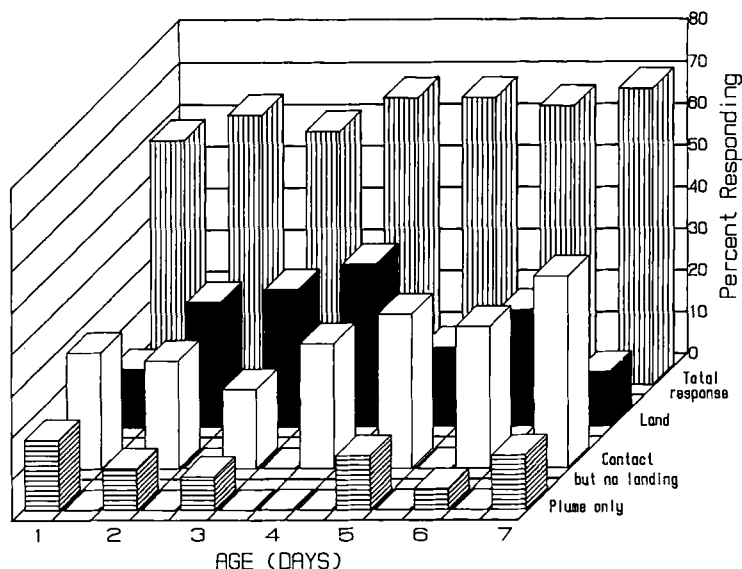


FIG. 2. Response in a flight tunnel of mated *Heliothis subflexa* females of indicated age to 1-g equivalent of an attractant extracted by washing fresh groundcherry leaves in methanol. Total response includes flying up the odor plume with no contact with the dispenser, contacts without landing, and landings.

TABLE I. FREQUENCY OF BEHAVIORAL RESPONSES EXHIBITED BY INDIVIDUAL, MATED *Heliothis subflexa* FEMALE MOTHS OF INDICATED AGE TO 1-GRAM EQUIVALENT OF ATTRACTANT FROM GROUNDCHERRY LEAVES DISPENSED DURING 120-SEC TESTS IN FLIGHT TUNNEL

Moth age (days)	Mean (\pm SE) No. contacts w/dispenser		Mean (\pm SE) No. plume trackings		
	Before landing ^a	No landing	Before landing	No landing	No contact or landing
1	1.0 \pm 0.0	2.2 \pm 0.9	2.5 \pm 1.5	1.6 \pm 0.3	1.3 \pm 0.3
2	2.3 \pm 0.2	2.1 \pm 0.2	1.3 \pm 0.1	1.4 \pm 0.2	1.9 \pm 0.1
3	3.0 \pm 0.6	4.8 \pm 1.1	1.3 \pm 0.2	1.7 \pm 0.4	1.9 \pm 0.6
4	2.0 \pm 0.5	1.5 \pm 0.3	1.4 \pm 0.2	1.5 \pm 0.3	
5	1.7 \pm 0.4	2.7 \pm 0.5	1.1 \pm 0.1	1.5 \pm 0.2	1.0 \pm 0.0
6	1.2 \pm 0.2	2.7 \pm 0.3	1.1 \pm 0.1	3.1 \pm 0.8	2.0 \pm 0.0
7	2.7 \pm 0.0	2.8 \pm 0.5	1.3 \pm 0.0	2.1 \pm 0.2	1.5 \pm 0.3

^aIncludes landing contact.

TABLE 2. FLIGHT DATA MEANS OF 3- TO 6-DAY-OLD, MATED *Heliothis subflexa* FEMALE MOTHS RESPONDING TO 1-GRAM EQUIVALENT OF ATTRACTANT FROM GROUNDCHERRY LEAVES DISPENSED DURING 120-SEC TESTS IN FLIGHT TUNNEL

Flight data	Behavioral response exhibited by moths (\pm SE)			
	None (27%)	Plume tracking (19%)	Contact with dispenser	
			Did not land (17%)	Landed (37%)
Seconds before flight initiation	13.2 \pm 1.7	8.3 \pm 1.1	8.8 \pm 3.1	11.3 \pm 1.0
Number of plume trackings	0	1.7 \pm 0.1	1.9 \pm 0.1	1.1 \pm 0.0 ^a
Duration of plume trackings (sec)	—	3.6 \pm 0.2	4.1 \pm 0.9	3.9 \pm 0.5
Number of contacts with dispenser	0	0.0	1.9 \pm 0.3	1.8 \pm 0.2 ^a
Total flight time (sec) ^b	80.7 \pm 9.2	101.4 \pm 3.3	107.1 \pm 3.1	38.5 \pm 3.3 ^a

^aFlight test terminated upon landing of moth on attractant dispenser.

^bMoths were in resting state for balance of 120-sec test.

assumed on the sides of the tunnel during each 120-sec test, the total flying times (80.7–107.1 sec) of females that flew randomly, flew up the plume, or contacted and did not land on the substrate were significantly longer than the 38.5 (\bar{X}) sec flown before landing by those that did land ($F = 36.25$, $df = 8$, $P < 0.0001$, ANOVA). Upon landing, ca. 50% of the moths landing were observed to oviposit on the dispenser before they could be captured and removed from the tunnel (Figure 1). However, detailed ovipositional behavior after landing was not studied in this experiment.

The HS females that landed on the extract-treated substrate in experiment 3 (Table 2) made significantly fewer flights upwind toward the source of the plume before landing than moths that responded to groundcherry extract by contacting or only following the plume ($F = 27.22$, 6 df , $P < 0.001$, ANOVA). However, the mean duration of the plume trackings was statistically equal among moths that landed, contacted, or plume tracked only. The mean number of contacts (1.9) by moths that made contact but did not land was almost the same as the number of contacts (1.8) made before and at time of landing by those that did land.

The data obtained from the 4-day-old moths ($N = 158$) in experiment 3 are presented in Figure 3 to illustrate the typical flight behavior of the mated females in response to groundcherry extract. Most (96%) of the HS females

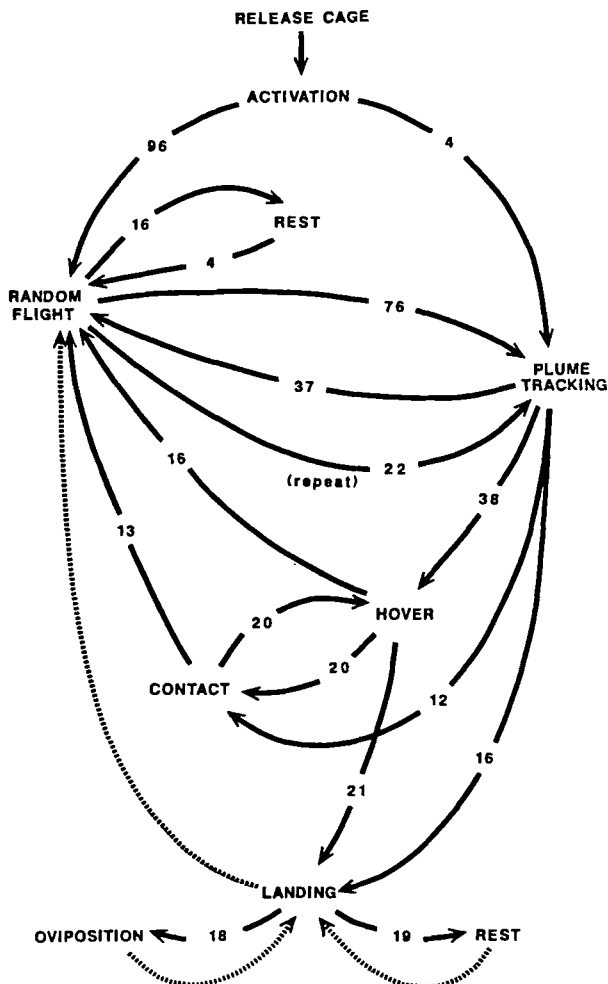


FIG. 3. Behavioral paths of mated female *Heliothis subflexa* females ($N = 158$) responding to 1-g equivalent of a methanol extract from whole-leaf washes of fresh groundcherry leaves. Values indicate percent of total insects responding. Broken lines indicate that observations were discontinued at the time of landing except for noting if oviposition occurred immediately.

began nondirectional (random) flight after emerging from the release cage at the downwind end of the flight tunnel. Random flight usually began in an upwind direction. A few of the moths (4%) began flying up the plume almost immediately upon release. Moths were more likely to begin flying up the plume trail

of the groundcherry extract if they remained in the release cage for a few sec before initiating flight.

During random flight, 76% of the HS moths detected and followed the extract plume (Figure 1), but of these, 37% returned to random flight (Figure 3). About half the moths that resumed random flight made additional flights up the plume. The moths usually began directed flight up the odor plume when they were 40–80 cm downwind from the extract dispenser. After flying up the plume, 38% of the moths began to hover ca. 5 cm from the dispenser. These moths then contacted (20%) or landed (21%) on the treated surface or returned to random flight. After making contact, they either returned to random flight or resumed the hovering position and then made a repeated contact or landed.

Females that followed the plume of the groundcherry extract, but did not hover near the dispenser, either contacted (12%) or landed (16%) without hesitation on the extract-treated substrate. More than one third of the moths flown in this experiment landed and either began to oviposit (18%) on the extract-treated substrate or assumed a resting position (19%). The significant ($P < 0.05$) behavior transitions, as determined by calculating chi-squared values for observed and expected frequency transitions to a behavior from the preceding behavior (Teal et al., 1981), are indicated on the flow diagram (Figure 4). After plume tracking, 32 of the 51 moths that contacted without landing hovered near the dispenser before making contact with the substrate, and 33 of the 58 moths that landed hovered before landing. The total positive response (landing, contacting, or plume tracking without making contact) was 80%.

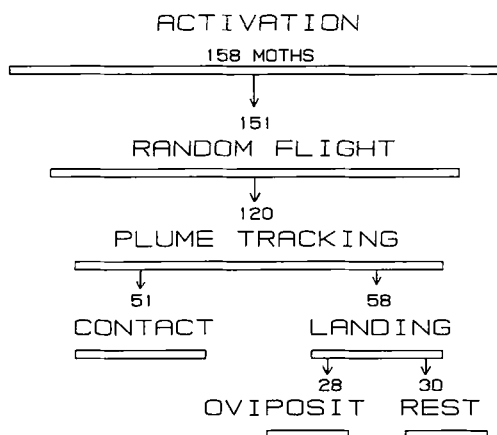


FIG. 4. Significant behavior transitions of mated *Heliothis subflexa* females responding to 1-g equivalent of methanol extract from whole-leaf waxes of fresh groundcherry leaves. Lengths of horizontal lines are decreased as transitions become less frequent.

There were no differences in behavioral responses to groundcherry extract by HS females that mated only once and those that had multiple matings. Of 392 females examined, 35% mated more than once before testing. The mean total positive response to the groundcherry extract was 73% for each group. Similar, but significantly less, positive response was observed when using virgin HS females ($\bar{X} = 41.7\%$) and males ($\bar{X} = 19.6\%$). Also, there were no significant differences among responses by 0-, 2-, and 4-day-old virgin moths with 28, 52, and 46%, respectively, demonstrating a positive response to the extract.

These results are particularly encouraging for future studies of insect-host plant relationships. The flight bioassay described demonstrates conclusively that volatile components attractive to HS can be extracted with a methanol wash of fresh whole groundcherry leaves. To our knowledge, this is the first documented demonstration of a flight response of female *Heliothis* moths to chemicals extracted from their host plant. This extract at dosages of 0.01–10.0 g equivalent elicited significant ovipositional response by HS when tested in an olfactometer as described previously (Mitchell and Heath, 1987). Characterization of the active chemicals and evaluation of the host-finding behaviors to these chemicals by HS \times HV hybrids are underway.

Although HS has a limited host range, there is reason to believe that polyphagous species also may be attracted to odors emanating from plants. A recent report by Rembold and Tabor (1985) showed that *H. armigera* (Hübner) females (a polyphagous species) responded differentially in oviposition trials to odors obtained by pulling air over seedlings of two cultivars of pigeonpea, *Cajanus cajan* L. Millsp. Jackson et al. (1984) reported that whole-leaf cuticular washes from green leaves of flue-cured tobacco stimulated HV oviposition when sprayed onto leaves of a resistant tobacco strain. It appears reasonable that using a flight bioassay of the type described here, alone or in combination with a behavioral oviposition bioassay (Mitchell and Heath, 1987), would add a new dimension to the study of insect-host plant resistance and enable integration of insect behavior and plant chemistry. Once a specific behavior (e.g., directed flight toward an odor source) is identified, the bioassay would permit the isolation of the active components. After the active material is isolated and characterized, the bioassay then could be used to screen other cultivars and even wild plants for germ plasm having none or substantially reduced levels of the active compounds. Programs of this type would reduce the screening time of plants and could be carried out year-round in a laboratory setting. Selected germ plasm could be tested in competitive field trials and then incorporated into commercial lines using classical or perhaps even the newer molecular genetic techniques. Such procedures conceivably would reduce significantly the time and expense of bringing resistant cultivars to market.

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INTER- AND INTRAPOPULATION VARIATION
OF THE PHEROMONE, IPSDIENOL
PRODUCED BY MALE PINE ENGRAVERS,
Ips pini (SAY)
(COLEOPTERA: SCOLYTIDAE)

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Abstract—We determined the chirality of ipsdienol in individual male pine engravers, *Ips pini* (Say), from New York, California, and two localities in British Columbia (BC). Both quantity and chirality of ipsdienol varied significantly between and within populations of *I. pini*. Beetles from California and southeastern BC produced primarily (*R*)-(–)-ipsdienol with mean ratios of (*S*)-(+) : (*R*)-(–) of 9:91 and 11:89, respectively, while beetles from New York produced primarily (*S*)-(+) ipsdienol with a mean (*S*)-(+) : (*R*)-(–) ratio of 57:43. A population from southwestern BC was unlike any other known western population, producing primarily (*S*)-(+) ipsdienol with a mean (*S*)-(+) : (*R*)-(–) ratio of 66:34. In contrast to the unimodal chirality profiles for ipsdienol production in populations from California and southeastern BC, the profiles of the populations from southwestern BC and New York were bimodal, with a common mode at approximately 44:56 (*S*)-(+) : (*R*)-(–). Bimodality in the profiles of ipsdienol chirality in two populations of *I. pini* and remarkably high levels of intrapopulation variation in pheromone chirality in all four populations suggest that evolutionary change in pheromone channels of communication could occur, possibly in response to artificial selection pressures such as mass trapping.

Key Words—*Ips pini*, Coleoptera, Scolytidae, aggregation pheromone, ipsdienol, geographic variation, intrapopulation variation, speciation.

INTRODUCTION

Pine engravers, *Ips pini* (Say) (Scolytidae: Coleoptera), are common and ubiquitous bark beetles found throughout North America. They breed primarily in the phloem tissue of the boles and branches of pines and can be a serious pest in stands of lodgepole, *Pinus contorta* var. *latifolia* Engelmann, and ponderosa pines, *P. ponderosae* Dougl. ex Laws. (Furniss and Carolin, 1980). Male *I. pini* are polygamous with harems of three to four females, on average. Each male excavates a nuptial chamber in the phloem of the host. Females in his harem then construct egg tunnels that radiate from the nuptial chamber, resulting in a characteristic X- or Y-shaped gallery, often engraved in the sapwood. The larvae feed in the surrounding phloem, pupate at the end of their feeding tunnels, and mature adults bore out through the bark and disperse to new hosts (Chamberlin, 1958; Bright and Stark, 1973; Bright, 1976; Furniss and Carolin, 1980; S.L. Wood, 1982).

Like many scolytids, *I. pini* aggregate rapidly and in large numbers to a suitable host (Anderson, 1948). Both sexes of *I. pini* are attracted to a terpene alcohol, ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Vité et al., 1972; Stewart, 1975). Only male *I. pini* produce ipsdienol, presumably from the host monoterpene, myrcene, as shown for male *I. paraconfusus* (Hughes, 1974; Byers et al., 1979; Hendry et al., 1980; Byers, 1981; Fish et al., 1984). Female *I. pini* are not known to produce any pheromone.

Geographic variation in the use of ipsdienol as a pheromone is known (Lanier, 1972). Ipsdienol exists as two optical isomers or enantiomers (Figure 1), differing only in the absolute configuration around the chiral center. Males from California (Stewart, 1975; Birch et al., 1980) and Idaho (Plummer et al., 1976) produce only (*R*)-(-)-ipsdienol while beetles from New York produce a 65:35 mixture of (*S*)-(+)- and (*R*)-(-) enantiomers (Lanier et al., 1980). California beetles are attracted by (*R*)-(-)-ipsdienol but are repelled by (*S*)-(+)-ipsdienol (Birch et al., 1980), while New York beetles respond best to a racemic mixture (equal quantities of (*S*)-(+)- and (*R*)-(-)-ipsdienol) (Lanier et al., 1980).

Pheromones are important in speciation and community structure in many Coleoptera and Lepidoptera, particularly in the families Scolytidae and Tortricidae, respectively (Lanier and Burkholder, 1974; Roelofs and Cardé, 1974; Cardé and Baker, 1984). In many Tortricidae, specificity in the use of pheromones is based on the ratio of *E* and *Z* isomers (Cardé and Baker, 1984), comparable to the specificity shown by bark beetles for enantiomeric ratios (Birch, 1984).

In order to understand if and how natural selection structures the use of pheromones, estimates of intrapopulation variation and heritability in pheromone traits are first required (Lanier and Burkholder, 1974; Collins and Cardé,

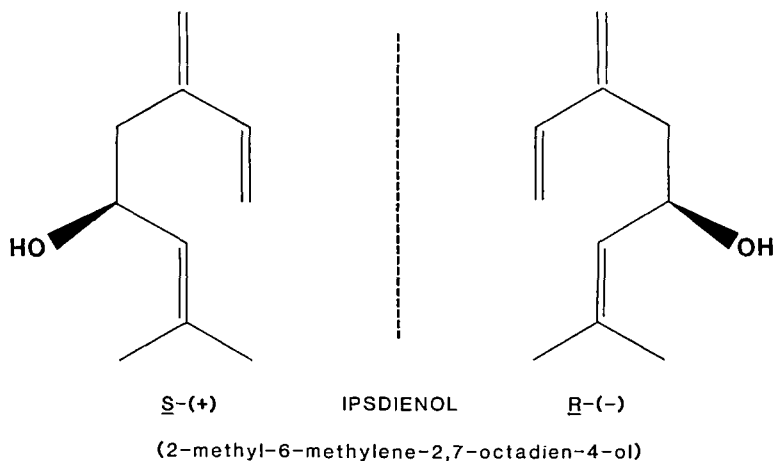


FIG. 1. Stereochemistry of the aggregation pheromone, ipsdienol, produced by male pine engravers, *Ips pini* (Say).

1985). To date, variation within populations of *I. pini* has been masked by the need for pooled samples of 300 or more beetles to obtain a sufficient quantity of ipsdienol for a single determination of chirality. It is now possible to determine the chirality of as little as 25 ng of ipsdienol by splitless gas chromatography following derivatization to acetyl lactate diastereomers (Slessor et al., 1985). By using this procedure we were able to elucidate the variation in quantity and chirality of ipsdienol within and between populations of *I. pini*.

METHODS AND MATERIALS

Collection of Pine Engravers. Populations of pine engravers were collected from four localities in North America. In 1984 we collected bolts of lodgepole pine infested with live broods of *I. pini* from Kimberley in southeastern British Columbia and just east of Manning Park in southwestern BC. Infested bolts were placed in rearing cages in the laboratory and adult beetles were collected after emergence as mature adults. In 1984 and 1985, newly emerged adults were transported by aircraft from Newcomb in New York, and Hat Creek in California. Red pine, *P. resinosa* Ait., was the brood host species for beetles from Newcomb; lodgepole pine was the brood host for beetles from the other three localities.

Procedure for Extracting and Analyzing Ipsdienol. 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 Man-

ning Park, BC. 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. These extracts were analyzed by splitless capillary gas chromatography (Hewlett Packard HP 5890 using a 30-m \times 0.25-mm ID fused silica column), before and after derivatization to acetyl lactate diastereomers (Slessor et al., 1985). Retention times of ipsdienol and its derivatives were determined with racemic ipsdienol obtained from Borregaard A.S., Sarpsborg, Norway, and chiral 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 (Hewlett Packard HP 5985B).

Procedure for Statistical Analyses. Coefficients of variation (Sokal and Rohlf, 1981) were calculated for quantity and chirality of ipsdienol using transformed data to obtain normality in the data sets. In addition to the mean chiral ratio for individuals, a pooled chiral ratio was also determined for each population using the pooled quantities of each enantiomer. The pooled chiral ratio is comparable to the method used in previous studies (Stewart, 1975; Plummer et al., 1976; Birch et al., 1980; Lanier et al., 1980). For each pooled chiral ratio, a root-mean-squared error (RMSE) was calculated using the following expression:

$$\text{RMSE} = (x_i - x_p)^2 + [\text{SE}(x_i)]^2$$

where x_i stands for the mean chirality for population i , x_p is the chiral ratio using pooled data for population i and $\text{SE}(x_i)$ is the standard error for the mean chiral ratio, x_i . Quantities were transformed by $\log(1 + X)$ while the data for chiralities were transformed by $\arcsin \sqrt{X}$. Student-Newman-Keuls test at $P < 0.05$ was used to compare mean quantities of ipsdienol produced by males between the four localities. Coefficients of correlation between quantity and chirality of ipsdienol were determined for each locality and adjusted for degrees of freedom. The Minitab Statistical Package (Statistics Department, The Pennsylvania State University, University Park, Pennsylvania 16802) was used for Student-Newman-Keuls test and the calculations of coefficients of correlation.

RESULTS

Quantity of Ipsdienol. The mean quantities of ipsdienol per male varied between populations of *I. pini* (Table 1), probably due to environmental factors and differences in vigor. Males from southeastern BC were quick to bore into logs and produced copious amounts of frass, while beetles from New York were the least vigorous of all the populations with respect to rates of boring and

TABLE 1. QUANTITIES AND CHIRALITIES OF IPSIDIENOL PRODUCED BY INDIVIDUAL MALE *Ips pini* (SAY) FROM 4 LOCALITIES IN NORTH AMERICA.

Locality	Quantity of ipsdienol (ng)			Chirality of ipsdienol (S)-(+):(R)-(-)			Correlations between quantity and chirality of ipsdienol ^c				
	N	Mean ± SE ^a	CV ^b	N	Mean ratio	SE	CV ^c	Pooled ratio	RMSE ^d	r	P value
New York	110	162 ± 32a	80.5%	55	56.9:43.1	1.3	13.2%	63.8:36.2	7.0	+0.47	<0.001
California	73	203 ± 33b	36.0	62	8.9:91.1	2.1	19.0	1.9:98.1	7.3	-0.32	<0.01
Southwestern BC	457	312 ± 16b	40.0	344	65.7:34.3	1.0	33.2	65.3:34.7	1.0	0.00	NS
Southeastern BC	158	522 ± 41c	26.4	173	10.8:89.2	1.4	19.8	5.1:94.9	5.9	-0.41	<0.001

^aMeans followed by a different letter are significantly different, $P < 0.05$.

^bCoefficients of variation (CV) for data transformed by $\log(1 + X)$.

^cCoefficients of variation (CV) for data transformed by $\arcsin \sqrt{X}$.

^dRoot-mean-squared error (see text).

^eCoefficients of correlation (r) were adjusted for degrees of freedom.

feeding. Geographic variation in brood host could have affected the ability of adult males to produce ipsdienol in lodgepole pine. New York beetles were bred in red pine, in contrast to beetles from the other three localities which were bred in lodgepole. Lodgepole used as a brood host could also vary significantly between localities. Beetles from southeastern BC were bred in logs of lodgepole pine with thicker phloem than logs used for beetles from southwestern BC.

Intrapopulation variation in the quantity of ipsdienol produced by individual males was found in all four populations (Figure 2A–D), with coefficients of variation ranging from 26.4 to 80.5% (Table 1). The high coefficient of variation for the New York population may be a consequence of the change from red pine, as a brood host, to lodgepole pine for pheromone production. Most males contained low quantities of pheromone and relatively few contained large amounts. The frequency distributions are similar to those for the production of *trans*-verbenol (*trans*-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol) by female *Dendroctonus ponderosae* Hopk. (Borden et al., 1986) and *cis*-verbenol and 2-methyl-3-buten-2-ol by male *I. typographus* (L.) (Birgersson et al., 1984; Schlyter et al., 1985).

Chirality of Ipsdienol. Variation in the chirality of ipsdienol was very evident between populations (Figure 2E–H). Males from California and southeastern BC produced primarily (*R*)-(–)-ipsdienol with mean (*S*)-(+) : (*R*)-(–) ratios of 9 : 91 and 11 : 89, respectively (Table 1). As expected, males from New York produced primarily (*S*)-(+) -ipsdienol with a mean (*S*)-(+) : (*R*)-(–) ratio of 57 : 43. However, the mean chiralities for all three of the above populations differ significantly (*t* tests, $P < 0.001$) from the previously published (*S*)-(+) : (*R*)-(–) ratios of 0 : 100 for California (Birch et al., 1980) and Idaho (similar to southwestern BC) (Plummer et al., 1976), and 65 : 35 for New York beetles (Lanier et al., 1980).

When the mean chiralities of ipsdienol in the same three populations were estimated from our pooled data (Table 1), the estimates did agree with the published estimates (*t* tests, $P > 0.05$). The bias in the estimator using pooled data is due to weak but significant correlations between the quantities and chiralities of ipsdienol in individual males (Table 1). However, the correlations are not consistent between the populations and change in magnitude and sign. Although ratio estimates on pooled samples are biased estimates of the true population means, they do reflect the overall chirality of ipsdienol produced by several hundred males on a single tree or log. The possibility exists that individuals attracted by the pooled pheromone from a large group of males may be favored differentially, by natural selection, over individuals attracted to the pheromone produced by an average male. Both methods of estimating chirality are valid but must be clearly stated and interpretation of the data related to the appropriate level of selection.

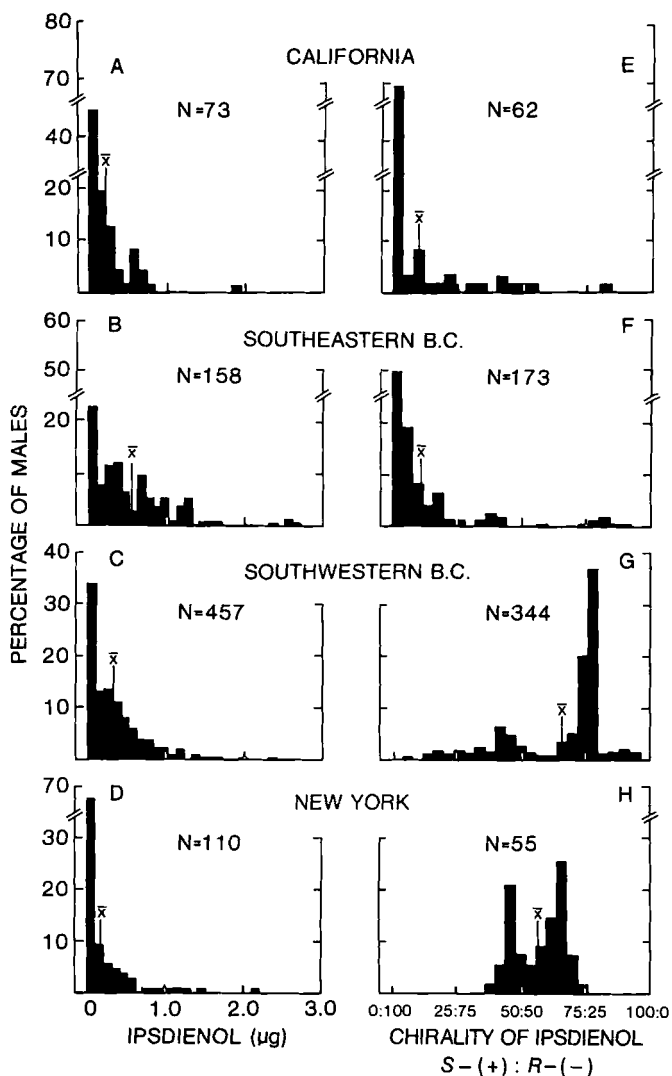


FIG. 2. Frequency distributions of the quantities (A-D) and the chiralities (E-H) of the aggregation pheromone, ipdienol, produced by male pine engravers, *Ips pini* (Say). Means are denoted by \bar{X} .

The population from southwestern BC (Figure 2G) is remarkably different from the other two western populations (Figure 2E, F). Males in this population produced primarily (*S*)-(+)-ipsdienol with a mean (*S*)-(+):(R)-(-) ratio of 66:34 (Table 1). This population negates previous generalizations that western populations of *I. pini* are homogeneous with respect to the chirality of their aggregation pheromone. Of the four populations studied, this is the only population in which there is no correlation between quantities and chiralities of ipsdienol in individual males (Table 1). Both mean and pooled estimators of chirality yielded the same result.

Intrapopulation variation in the chirality of ipsdienol was found in all four populations (Figure 2E-H) with coefficients of variation ranging from 13.2% to 33.2% (Table 1). Variation in the modalities of the four distributions of the chirality of ipsdienol is also apparent. The distributions for the populations from California and southeastern BC have long tails but are strongly centered around an (*S*)-(+):(R)-(-) ratio between 5:95 and 15:85, with only one mode in each. Bimodality is evident in the New York population, with both modes almost equal in size. In the population from southwestern BC, the modes are distinctly different in size with the major mode between 75:25 and 80:20 (*S*)-(+):(R)-(-). Both New York and southwestern BC populations have modes between 40:60 and 45:55 (*S*)-(+):(R)-(-). The earlier estimates from pooled samples (Stewart, 1975; Plummer et al., 1976; Birch et al., 1980; Lanier et al., 1980) failed to disclose these striking differences between individuals in the same population as well as the bimodality in some populations.

DISCUSSION

In bark beetles, a pheromone message should be a reliable indicator of either host quality or the genetic quality of the sender. This message should not be subject to chance variation or noise. In *I. pini*, we found that there is substantial variation in both quantity and chirality of ipsdienol. Most of the variation in quantity can probably be attributed to variation in vigor and environmental factors, such as brood host and levels of precursors in the host tissue. Production of the pheromone, *cis*-verbenol, by male *I. paraconfusus* increased directly with the concentration of vapors of the precursor, (-)- α -pinene (Byers, 1981). In *I. typographus*, over 80% of the variation in quantities of *cis*-verbenol, *trans*-verbenol, and myrtenol were explained by the variation in the amounts of α -pinene in the host (Birgersson et al., 1984). In addition, the quantity of pheromone in the hindgut may vary over time in the same individual. The rates of ingestion and defecation may not be constant.

The major factors responsible for the variation in chirality of ipsdienol are not the same as those responsible for the variation in quantity. No more than

25% of the variation in chirality of ipsdienol in any population was explained by the variation in the quantity of ipsdienol (all $r^2 < 0.25$). In southwestern BC less than 1% of the variation in the chirality of ipsdienol was explained by the variation in quantity of ipsdienol ($r^2 < 0.01$). Since both enantiomers are produced from the same achiral precursor, myrcene (Hughes, 1974; Byers et al., 1979; Renwick and Dickens, 1979; Hendry et al., 1980; Byers, 1981; Fish et al., 1984), it seems unlikely that environmental factors should significantly affect the chirality of ipsdienol, certainly not to the extent seen in the quantity of ipsdienol. Variation in enzymatic composition due to genetic variation is the most probable source of the variation in chirality.

Our data are consistent with the hypothesis that the production of ipsdienol of a specific chirality by an individual male *I. pini* is a quantitative genetic trait. We are currently trying to quantify the variation in behavioral responses associated with ipsdienol. We plan to determine the heritability of both the production of and the responses to chiral ipsdienol in individual beetles. To date, heritability of pheromone quality has been clearly estimated only for the pink bollworm, *Pectinophora gossypiella* (Saunders). Collins and Cardé (1985) found that the heritability of the sex pheromone blend of the *Z,E* and *Z,Z* isomers of 7,11-hexadecadienyl acetate, produced by female *P. gossypiella* from a laboratory strain was 0.34. The variation of the chirality of ipsdienol in *I. pini* (Table 1) is more than that of the *E:Z* ratio of the sex pheromone in *P. gossypiella* ($CV = 5.3\%$). Assuming that there is a large heritable component to the variation in ipsdienol chirality, then such high levels of variation should facilitate microevolutionary changes in relatively short periods of time, possibly giving rise to further geographic variation in *I. pini*.

In addition, bimodality in the populations from New York and southwestern BC suggests that distinct groups exist within populations and that disruptive selection may be occurring in these areas. The modes may separate further and possibly result in behavioral isolation and subsequent speciation. Alternatively, bimodality may be stable in these populations, representing mixed evolutionary stable strategies (Smith, 1982) in which individuals from both modes have equal fitness, on average.

Knowledge of the variation and heritability in the use of pheromones should help predict the consequences of artificial selection pressures such as mass trapping. Bark beetles are major economic pests of forestry (Furniss and Carolin, 1980), and pheromones are gaining acceptance as pest management tools. If pheromones are used to mass trap *I. pini*, would "resistance" to pheromone baits occur? Lanier et al. (1972) suggested that some populations of *I. pini* are already resistant to a single pheromone blend. Since the levels of variation of ipsdienol chirality were high in all four populations of *I. pini* (Table 1), we hypothesize that resistance to a pheromone blend could develop within a population as well. The development of resistance has been shown in laboratory

colonies of the khapra beetle, *Trogoderma granarium* Everts. After 18 generations of selection for nonresponse by males, there was a 74% reduction in mean response by males to the natural pheromone produced by female beetles (Rahalkar et al., 1985). The possibility exists that populations of bark beetles, such as *I. pini*, subjected to repeated use of pheromone-based trapping programs using a fixed pheromone blend, could develop resistance by shifting to another pheromone blend (Lanier et al., 1972; Lanier and Burkholder, 1974).

Competition for Pheromone Channels of Communication. An understanding of the mechanisms of any behavior must go along with an understanding of the functional value of that behavior. With respect to any insect using pheromones, we need to ask why a pheromone blend exists as well as how. Selection should favor individuals that use a communication system, such as pheromones, that minimizes the expenditure of energy and time (Matthews and Matthews, 1978). The pheromone blends used by different insect species can be called pheromone channels of communication (Cardé and Baker, 1984). Each channel is comprised of a cluster of points in an n -dimensional space. Each axis, in this space, is an array of quantities of a single and unique semiochemical. The coordinates of each point represent one possible pheromone blend that has a probability, greater than zero, of successfully communicating the pheromone message. The density of points varies within the cluster. The highest density is located centrally and represents the optimal pheromone blend for communication. In tortricid moths, the dimensions of the pheromone channels consist of a variety of 12- and 14-carbon-chain-length acetates, aldehydes, and alcohols, and their isomers when double bonds are present along the carbon chain (Cardé and Baker, 1984).

Bark beetles use pheromones to transmit information about location to conspecifics, usually to facilitate mating or feeding. In British Columbia, 82 scolytid species have been found either on or in lodgepole pine; at least 56 species breed in lodgepole pine (Bright, 1976; S.L. Wood, 1982; Evans, 1983). Although most of these species have not been investigated for the use of pheromones, the ubiquitousness of pheromone communication in the Scolytidae (Klassen et al., 1982) suggests that such communication should occur in many of the 56 species breeding in lodgepole pine. The number of potential competitors for pheromone channels of communication just among bark beetles is very high. In addition, there are insects in 10 other orders that are known to use pheromone communication (Klassen et al., 1982), many of which have representatives in stands of lodgepole pine (Furniss and Carolin, 1980). If the number of channels, particularly those with the best physiochemical traits for communication, is limited, then competition for pheromone channels should occur, particularly if the channels also differ in quality as a communication system.

If competition does occur, then three major predictions can be made.

Firstly, sympatric species of bark beetles should use separate and distinct pheromone channels in order to minimize competition for a resource or to maintain species specificity in mating (D.L. Wood, 1970; Lanier and Burkholder, 1974; Cardé and Baker, 1984; West-Eberhard, 1984). In California, the pheromone channel for *I. pini* is comprised primarily of (*R*)-(-)-ipsdienol (Birch et al., 1980; Plummer et al., 1976) (Figure 2E). The channel for the sympatric species, *I. paraconfusus*, is a blend of (*S*)-(+)-ipsdienol, (*S*)-*cis*-verbenol and (*S*)-(-)-ipenol (Silverstein et al., 1966a,b; D.L. Wood et al., 1967, 1968).

Secondly, if competition is structuring the use of pheromones, then the channels should not only be distinct, they should also be widely separated (Matthews and Matthews, 1978; Cardé and Baker, 1984). Widely separated signals (i.e., signals with high signal-to-noise ratios) minimize the possibility of making a costly mistake. The cost may involve loss of time or energy or may be a higher risk of predation because of greater exposure time before finding a host or mate.

In California *I. pini* and *I. paraconfusus* seem to have separated their channels almost maximally with respect to the chirality of ipsdienol and with respect to the presence of ipenol (Light and Birch, 1977). *I. pini* produces primarily (*R*)-(-)-ipsdienol (Plummer et al., 1976) (Figure 2E) and response is inhibited by ipsdienol when the percentage of the (*S*)-(+)-enantiomer is greater than 5% (Birch et al., 1980). Most male *I. paraconfusus* produce ipsdienol with a chiral ratio probably between 90:10 (Silverstein and Young, 1976) and 95:5 (*S*)-(+):(*R*)-(-) (Stewart, 1975). Response of *I. paraconfusus* to ipsdienol is inhibited when the chirality is predominantly (*R*)-(-) (Light and Birch, 1977; Birch et al., 1980). *I. paraconfusus* also uses (*S*)-(-)-ipenol as part of its pheromone channel (Silverstein et al., 1966a,b; D.L. Wood et al., 1967, 1968). (*S*)-(-)-Ipsenol inhibits response of *I. pini* to (*R*)-(-)-ipsdienol (Birch and Wood, 1975; Birch and Light, 1977; Birch et al., 1977).

Lastly, one should expect geographic variation in the specificity and separation of pheromone channels due to geographic differences in competition pressures (Cardé and Baker, 1984). Competition pressure should increase with either an increase in the absolute number of scolytid species or an increase in the number of superior competitors. In areas where the pressure is lower, the channels should be either different or broader. In California *I. pini* is sympatric with *I. paraconfusus* and uses primarily (*R*)-(-)-ipsdienol (Birch et al., 1980) (Figure 2E). In New York where *I. paraconfusus* is absent (S.L. Wood, 1982), *I. pini* produces ipsdienol with (*S*)-(+):(*R*)-(-) ratios close to 50:50 (Figure 2H). Furthermore, the variation in chiral ratios is greater in the New York population than in the population from California (Table 1).

These three predictions are fairly straightforward; however, not all of our evidence appears to support them. For example, separation of all channels does not seem maximal. In 15 species of *Ips* studied to date, males in 14 species

produce ipsdienol and males in 10 produce ipsenol (Vité et al., 1972; Lanier and Burkholder, 1974; Francke et al., 1980; Borden, 1982; D.L. Wood, 1982). Three species of *Pityokteines* use ipsenol and two use ipsdienol (Harring, 1978). Ipsdienol is produced by males in four species of *Dendroctonus* following exposure to myrcene vapors (Hughes, 1973; Renwick et al., 1976; Byers, 1982; Hunt et al., 1986). One possibility is that physiochemical constraints limit the dimensions of pheromone channels to very few compounds, such as ipsenol and ipsdienol (Silverstein, 1977; Cardé and Baker, 1984). Another possibility is that parsimony of semiochemicals is beneficial (Blum, 1970, 1977). One compound may serve as a pheromone in various ways, or as an allomone or a kairomone, depending on the context in which it is used. The advantage may be a saving in production or receptor hardware without giving up the potential for a diversity of messages (Matthews and Matthews, 1978).

Another area of uncertainty regarding our predictions is that existing geographic differences are not all easily explained, nor do differences necessarily exist when they should, according to our predictions. *I. pini* is sympatric with *D. brevicomis* and *D. ponderosae* in both southwestern and southeastern BC (S.L. Wood, 1982), yet populations of *I. pini* from these two regions produce ipsdienol with different chiral ratios (Figure 2F, G). *I. paraconfusus* is not found in Canada (Bright, 1976), but the distribution of chiral ratios for *I. pini* is not much different between southeastern BC and California (Figure 2E, F). It is possible that some of the geographic variation may be a product of colonization of *I. pini* from different refugia following the last Ice Age.

Clearly, there is a need for more baseline information on the use of semiochemicals by all competing species in a given habitat before the selection pressures can be elucidated. An understanding of the population genetics in the use of pheromones should facilitate the testing of quantitative predictions about the various selection pressures affecting pheromone production and response and elucidate the importance of competition in structuring communities of bark and ambrosia beetles (Sturgeon and Mitton, 1982). Our data on the variation of pheromone production in *I. pini* constitute only one step towards understanding the mechanism structuring pheromone channels of communication. The hypothesis that competition is the major driving force in the structuring of pheromone channels in communities of bark beetles is still the most supportable, but the effects of competition may be dampened by benefits of parsimony of semiochemicals, in which many species share certain semiochemicals for differing reasons (Blum, 1970, 1977).

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FUSARIC ACID
A Secondary Fungal Metabolite that Synergizes Toxicity of
Cooccurring Host Allelochemicals to the Corn Earworm,
Heliothis zea (Lepidoptera)¹

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Abstract—The ability of naturally occurring levels of the *Fusarium* spp. fungal metabolite fusaric acid to synergize the toxicity of the allelochemicals gossypol, a saponin, and 6-methoxy-2-benzoxazolinone to larvae of *Heliothis zea* (Boddie) was tested. Levels of fusaric acid comparable to those found near the fungus increased mortality of *H. zea* to gossypol, the saponin, and 6-methoxy-2-benzoxazolinone, and decreased the development rate of surviving larvae exposed to gossypol and 6-methoxy-2-benzoxazolinone. Some effect was also noted for levels of fusaric acid found generally distributed throughout infected plants. The chemical properties of fusaric acid suggest that it synergizes the toxicity of the allelochemicals by inhibiting oxidative enzymes responsible for detoxification. Production of the biosynthetically simple fusaric acid may be a fungal strategy for conserving resources as compared to those fungi that produce biosynthetically complex toxins of their own.

Key Words—Fusaric acid, corn earworm, *Heliothis zea*, Lepidoptera, Noctuidae, saponin, gossypol, 6-methoxy-2-benzoxazolinone, synergism, allelochemical, mycotoxin.

¹The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

INTRODUCTION

Many secondary plant metabolites are thought to act as defensive chemicals to protect the plant from herbivores. Fungi also produce a variety of toxic chemicals that are thought to play a defensive role (Wicklow, 1984). Plants may contain additional chemicals that are thought to act as synergists of the cooccurring allelochemicals (Berenbaum, 1985); such synergism has been demonstrated in some instances (Berenbaum and Neal, 1985). These synergistic chemicals appear to act by inhibiting the enzymes responsible for detoxification of the allelochemicals by the herbivores (Berenbaum and Neal, 1985). Fungi also appear to possess secondary chemicals that can act as synergists of mycotoxins, again presumably by inhibiting enzymes responsible for detoxification (Dowd, 1988).

The fungal secondary metabolite fusaric acid is produced by many species of *Fusarium*, some of which also produce trichothecene mycotoxins (Marasas et al., 1984), which are toxic to insects (Wright et al., 1982). Other species of *Fusarium* (i.e., *Fusarium moniliforme*) do not produce mycotoxins that are toxic to insects (Marasas et al., 1984). Although fusaric acid is thought to be involved in the pathogenic process (Davis, 1969), this involvement has been disputed (Kuo and Scheffer, 1964). However, fusaric acid is known to inhibit metal-containing oxidative enzymes (Jain, 1982), which are similar in structure to the unspecific monooxygenases (EC 1.14.14.1; I.U.B., 1984) that are thought to be widely involved in the metabolism of xenobiotics by insects (Brattsten, 1979). This information suggests that fusaric acid may be able to act as a synergist of allelochemicals that may already be present in the host. Since both *Fusarium* spp. (Booth, 1971) and *Heliothis zea* (Boddie) (Metcalf et al., 1962) may compete for the same hosts (such as legumes, cotton, and maize), allelochemicals from these hosts were selected for testing in combination with fusaric acid at naturally occurring levels.

METHODS AND MATERIALS

Insects. Neonate larvae of *H. zea* were used for all assays. They were obtained from a laboratory colony reared on pinto bean-based diet (Dowd, 1987) at $27 \pm 1^\circ\text{C}$, $40 \pm 10\%$ relative humidity, and a 14 : 10 light-dark photoperiod.

Chemicals. The three test chemicals, gossypol, a saponin, and 6-methoxy-2-benzoxazolinone (MBOA) were obtained from Sigma Chemical Co., (St. Louis, Missouri), while fusaric acid was obtained from Aldrich Chemical Co. The saponin was from *Gypsophila* root (Sigma Chemical Co., personal communication) and is presumably related to the gypsoside reported from *Gypso-*

phila pacifica root (Kochetkov et al., 1963). All other chemicals were of reagent grade.

Diet Preparation. The pinto bean-based diet was prepared and added in 5-ml quantities to test tubes. The test tubes were held at 60°C until chemicals were incorporated to prevent solidification of the diet. The three allelochemicals were added in 125 μ l of acetone or water to the liquid diet to give a final concentration of 250 ppm, levels which are similar to naturally occurring levels (gossypol, Hedin et al., 1973; MBOA and derivatives, Manuwoto and Scriber, 1982; saponins, Applebaum and Birk, 1979). Fusaric acid was added in 125 μ l of water so that the final concentration approximated that found throughout infected plants (25 ppm, Gaumann, 1957), or a level that would be expected in the proximity of the fungus (250 ppm, J.D. Miller, personal communication). The chemicals were incorporated into the diets by blending vigorously with a vortex mixer for 20 sec. Preliminary observations with colored solutions of both water and acetone indicated uniform incorporation by this method. The diets were dispensed into culture plates and allowed to cool to room temperature. To remove the potentially toxic acetone, the diets were placed in a fume hood for ca. 20 min until slight darkening occurred. All diets were treated in the same way. The diets were cut into approximately equal sections, and each section was placed into a well of a 24-well immunoassay plate. A single neonate *H. zea* larvae was added to each well. To prevent desiccation of the diet, the plate was covered by a sheet of parafilm, a sheet of cardboard, and the plastic cover. The cover was secured by two rubber bands, and groups of plates were placed in two polyethylene bags held closely by rubber bands. The plates were held under the same conditions used to rear the insects. Mortality was checked at two, four, and seven days, and the surviving larvae were weighed after seven days. Each chemical set was tested on a total of 40 larvae. Mortalities were tested for synergistic interactions by log likelihood ratio tests using Yate's correction for small sample sizes, and weights were tested for synergistic interactions by factorial analysis (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

The toxicities of all of the allelochemicals were synergized when they cooccurred with fusaric acid at 250 ppm (Table 1). The toxicity of MBOA and gossypol (both mortality and weight reduction) was synergized to the greatest extent, while only the mortality due to saponin was increased by fusaric acid. A slight synergistic reduction in weight of the larvae was noted when gossypol was incorporated with the lower concentration of fusaric acid. Thus, the *Fusarium* species that produce fusaric acid should be able to synergize the toxicity of plant allelochemicals to the insects that feed on plants both organisms

TABLE I. EFFECT OF FUSARIC ACID ON TOXICITY OF PLANT ALLELOCHEMICALS TO *HELIOTHIS ZEA*

Chemical	Mortality (%)			7-day weight		
	-FA	FA1	FA10	-FA	FA-1	FA-10
Control	0.0	0.0	0.0	37.5 ± 1.5	34.2 ± 2.1	31.3 ± 2.3
MBOA	0.0	0.0	18.9*	26.7 ± 2.1	30.5 ± 2.0	14.0 ± 2.4
Gossypol	2.5	0.0	18.4*	33.0 ± 2.1	28.5 ± 2.1	15.7 ± 2.2*
Saponin	2.5	0.0	10.5	28.4 ± 2.1	25.6 ± 1.7	24.2 ± 2.1

"Mortality values are based on two studies of 20 insects each, while 7-day weights are based on the survivors of the mortality studies (means ± standard errors). MBOA = 6-methoxy-2-benzoxazolinone, FA1 = fusaric acid at 25 ppm, FA10 = fusaric acid at 250 ppm. Mortality values followed by * are significantly greater than predicted additive values at $P < 0.05$ when analyzed by the log-likelihood ratio test, with Yate's correction for small sample sizes (Sokal and Rohlf, 1969). Weights followed by * represent a significant interaction between the toxin and fusaric acid at $P < 0.05$ when analyzed by factorial analysis (Sokal and Rohlf, 1969). Some escapes of larvae occurred, and mortality values have been adjusted accordingly.

use as hosts, although the effect would be greater near the fungus, where the concentration of fusaric acid is higher. The enhanced toxicity of the chemical combinations could also deter feeding under field conditions.

Chelators such as fusaric acid bind to metal ions by contributing a free electron pair (Mellor, 1964). Chemicals that inhibit the unspecific monooxygenases by complexing with the iron atom present in these enzymes also must contribute a free electron pair (Ullrich and Duppel, 1975). Thus, the chemical properties of fusaric acid, coupled with its proven ability to inhibit other metalloxygenases (Jain, 1982), suggest that it is able to act as a synergist by inhibiting the unspecific monooxygenases of *H. zea* that are normally responsible for detoxifying the allelochemicals. For example, all three allelochemicals tested contain double bonds and other sites that can be attacked by unspecific monooxygenases from insects (Ahmad et al., 1986). Since plants also contain similar enzymes responsible for xenobiotic metabolism (Cole, 1983), fusaric acid could interact with these enzymes as well as part of the pathogenic process.

Fusaric acid is relatively simple for the *Fusarium* spp. to produce (Hill et al., 1966), in contrast to the highly toxic trichothecenes, which are produced by other species of *Fusarium* (Tamm and Breitenstein, 1980). Thus, the species of *Fusarium* that produce fusaric acid can conserve their resources by enhancing the preexisting defenses of the host plant to protect themselves, and potentially parts of the host plant remote to the infection, from consumption by insects such as *H. zea*. In cases where the infection by these *Fusarium* spp. is asymptomatic, the net protective effect could be similar to the acquired defenses pre-

viously reported for some plants that contain fungal endophytes. Although the ability of fungus-infected plants to deter insect feeding has been attributed to the induction of phytoalexin production in the infected plants (Karban et al., 1987), the toxicity of mycotoxins produced by pathogenic fungi themselves to insects has already been demonstrated in several instances (Wright et al., 1982). The present study indicates that chemicals produced by plant pathogenic fungi can also enhance the toxicity of preexisting plant defenses to insects.

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PRESENCE OF LONG-CHAIN DIALKYL ETHERS IN CUTICULAR WAX OF THE AUSTRALIAN CHRYSOMELID BEETLE *Monolepta australis*¹

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Abstract—Investigation of the lipid extract of the Australian chrysomelid beetle, *Monolepta australis*, has revealed a novel homologous series of long-chain, unsaturated-saturated dialkyl ethers in the cuticular wax. Gas chromatography-mass spectrometry, proton magnetic resonance, infrared spectroscopy, and chemical degradation have shown that ethers of formula $\text{CH}_3(\text{CH}_2)_9\text{CH}=\text{CH}(\text{CH}_2)_6\text{O}(\text{CH}_2)_{12-16}\text{CH}_3$ predominate.

Key Words—*Monolepta* beetle, red-shouldered leaf beetle, *Monolepta australis*, lipid extract, unsaturated aliphatic ethers, 7-octadecenyl alkyl ethers, gas chromatography-mass spectrometry, Coleoptera, Chrysomelidae.

INTRODUCTION

Long-chain, saturated aliphatic ethers have recently been reported from the cuticular waxes of the locust *Locusta migratoria cinerascens* (Genin et al., 1987). Concurrently we isolated and orally reported a homologous series of long-chain, unsaturated-saturated dialkyl ethers from the indigenous monolepta or red-shouldered leaf beetle, *Monolepta australis* (Jacoby).

M. australis is a chrysomelid beetle approximately 6 mm in length endemic to the subtropical central coast of eastern Australia. Swarms of the beetle now attack the foliage, flowers, and fruit of avocado, macadamia, lychee, mango, stone fruit, citrus, and eucalyptus windbreak species (Murray, 1982). Present methods of monitoring and control could be greatly improved by the use of

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pheromone-based traps. Consequently, the chemical constituents of *M. australis* were extracted and fractionated and are currently being purified and evaluated for pheromone-type activity. This first paper reports the isolation and identification of a novel series of long-chain ethers from the lipid fraction of the beetle.

METHODS AND MATERIALS

Swarms of *M. australis* (1.5 kg, approx. 93,000) were collected from ornamental trees at Wollongbar, New South Wales (28°50'S, 153°25'E) in summer and immediately soaked in doubly distilled petroleum spirit (bp 50–70°C May and Baker). After 20 days, the beetles were filtered, washed (2 × 1 liter, 5 min), soaked (2 liters, 14 hr), filtered, washed (2 × 1 liter) with petroleum spirit and the combined solutions concentrated to give a yellow wax (21.5 g, 1.4%). The washed beetles were then extracted at 25°C with dichloromethane and acetone for the investigation of more polar constituents. The yellow wax (21.5 g) was preabsorbed onto alumina (40 g) and subject to flash liquid chromatography on alumina (160 g).

Elution with aliquots (100 ml) of petroleum spirit (50–70°C) gave first a colorless wax fraction (IR 720 cm⁻¹) (0.62 g) followed by an orange-colored oil (3.10 g) showing some carbonyl (1740 cm⁻¹) and ether (1100–1200 cm⁻¹) infrared absorption. This second fraction was also preabsorbed on alumina (20 g) and subjected to flash chromatography on alumina (100 g). Elution with aliquots (50 ml) of light petroleum gave further wax (0.86 g) from fractions 1 and 2 and then an ether mixture (0.45 g) (IR 1118 cm⁻¹) from fractions 3–5. Thin-layer chromatography on silica gel when run in 4% ethyl acetate in petroleum spirit gave a single spot (R_f 0.47). This mixture distilled at 220°C at 0.5 mm. [¹H]NMR indicated an olefinic triplet at δ 5.35 (J 5.5 Hz, 2H), an oxymethylene triplet at δ 3.39 (J = 8.2 Hz, 4H), a methyl triplet at δ 0.88 (J = 7.0 Hz, 6H), and a prominent methylene signal at δ 1.26 (46–50 H). Signals for β -oxy and allylic methylenes were evident at δ 1.56 (4H) and δ 2.01 (4H), respectively.

Analytical gas chromatography was conducted on a Perkin Elmer Sigma 2B chromatograph using a 10-m × 0.22-mm ID, BP1-coated, fused-silica, open-tubular (FSOT) capillary column using N₂ as carrier gas. Individual runs were temperature programmed from 100°C to 300°C with no initial holding period. Percentage compositions were computed with a Perkin Elmer Sigma 10B data station, and Kovats indices were calculated with respect to straight-chain hydrocarbon standards.

Preliminary mass spectral data were obtained on an AEI MS 12 mass spectrometer at 70 eV ionizing voltage, 8000 V accelerating voltage with ion source at 200°C.

Combined gas chromatography-mass spectrometry was conducted on a Hewlett Packard MSD instrument using on-column injection into a 12-m \times 0.2-mm ID BP1 FSOT capillary column. Temperature was programmed to rise from 30°C to 220°C at 30°C/min and then to 300°C at 4°C/min.

Proton magnetic resonance spectra were recorded at 300 MHz in CDCl_3 on a Bruker CXP 300 spectrometer.

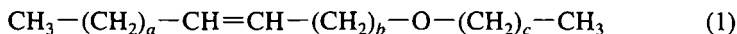
Chemical Degradation. The *M. australis* lipid ether fraction (1) (65.5 mg) in dry diethyl ether (2.0 ml) was treated with 3-chloroperbenzoic acid (46.8 mg, approx. 2 equiv.) at 25°C in the dark. After 64 hr, the solution was diluted with diethyl ether (20 ml), washed with: (1) 5% sodium bisulfite solution (5 ml), (2) 5% sodium bicarbonate solution (2 \times 5 ml), and (3) saturated sodium chloride solution (5 ml). The epoxide (5) solution was dried (Na_2SO_4) and without concentration stirred at 25°C with freshly prepared periodic acid (178 mg) (Willard, 1939). After 1 hr the mixture was diluted with diethyl ether (10 ml), washed with: (1) 10% sodium thiosulfate solution (5 ml), (2) saturated sodium chloride solution (5 ml), and dried (Na_2SO_4). Without concentration, the aldehyde mixture [6 + 7 (see Scheme 2 below)] was treated with lithium aluminium hydride (50.0 mg) and stirred. After 16 hr, excess reagent was decomposed by the dropwise addition of 10% ammonium chloride solution, dried (Na_2SO_4), filtered, and concentrated to give a colorless mixture of alcohols 8 + 9 (45.7 mg). This alcohol mixture was dissolved in dry chloroform (2.0 ml) and refluxed with excess trimethylsilyliodide (0.2 ml, 281 mg). After 1 hr, the mixture was cooled and decolorized by the dropwise addition of 10% sodium thiosulfate solution, diluted with diethyl ether, dried (Na_2SO_4), and concentrated. The resulting iodide mixture, 10 + 11 + 12, in dry diethyl ether (2.0 ml) was added to lithium aluminium hydride (103.6 mg) suspended in dry diethyl ether (1.0 ml) and stirred at 25°C. After 16 hr excess reagent was destroyed by the cautious dropwise addition of saturated ammonium chloride solution. The solution was dried (Na_2SO_4) and concentrated at 25°C to give the hydrocarbon mixture 13 + 14 + 15. All reactions were followed to completion using TLC, GLC, and IR spectroscopy. Hydrocarbon products 13, 14, and 15 were also confirmed by GC coinjection of authentic standards.

RESULTS AND DISCUSSION

The aliphatic ethers in *M. australis* comprise in excess of 2% of the total lipid fraction and in excess of 300 ppm of the entire fresh insect. Their presence in the cuticular wax layer was established by isolating the ether fraction from a brief (10 min), cold (0°C) extraction.

Identification was based on IR and NMR spectroscopy, gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS). IR absorption at 1118 cm^{-1} , when coupled with retention data from thin-layer, liquid,

and gas chromatography clearly indicated aliphatic ethers. GC indicated a mixture containing nine components, each contributing more than 1% to the total ether fraction (Figure 1). From the retention index data, the major (60%) component (Figure 1, peak 3) seemed to represent the middle member of a five-member homologous series. Traces of members of this series preceding peak 1 (Figure 1) were also evident from the GC and MS data. $[^1\text{H}]\text{NMR}$ indicated $-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ ($\times 1$), $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-$ ($\times 1$), $-\text{CH}_2-$ ($\times 23-25$) and $-\text{CH}_2-\text{CH}_3$ ($\times 2$), suggesting a long-chain ether series with formula:



GC-MS analysis defined each member of the series more specifically (Table 1). Molecular ion peaks were prominent for all except peak 6. All components gave strong fragments at m/z 250 and 281, representing fragment ions 2 and 4, resulting, respectively, from C-O and α -C-C cleavage (Scheme 1). The alternative α -C-C fragmentation was evident from prominent mass spectral peaks at $M-267$ in all members of the series except chromatographic peak 2. The alternative fragmentation in the unsaturated chain was absent. Support for this assignment is seen in the typical $\text{C}_n\text{H}_{2n+1}$, $\text{C}_n\text{H}_{2n-1}$, and $\text{C}_n\text{H}_{2n-2}$ decomposition fragments in the mass spectra. Thus an octadecenyl structure for the unsaturated half of the ether is established. The position of the double bond in the

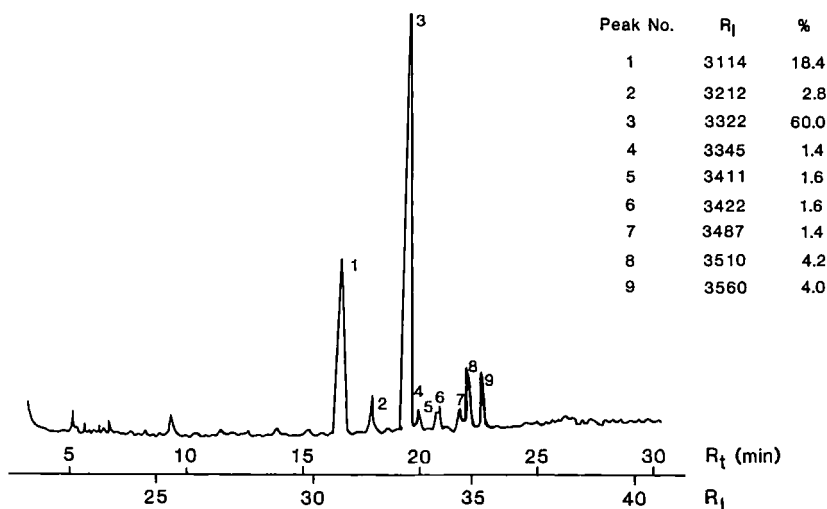
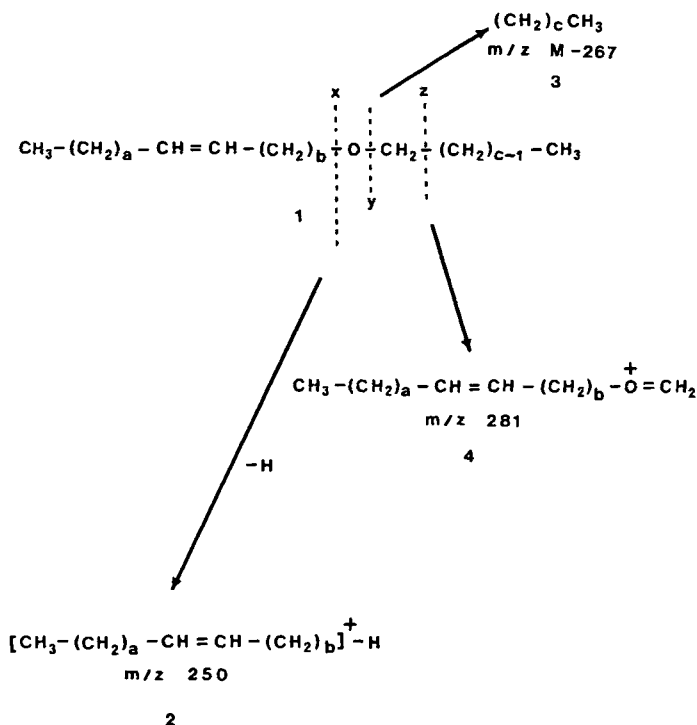


FIG. 1. The total ion current gas chromatogram of the long-chain ether fraction of *Monolepta australis* extract showing retention time (R_t) and retention index (R_i) axes and percentage contribution of major components.



SCHEME 1.

TABLE 1. MASS SPECTRAL INTENSITIES OF SIGNIFICANT PEAKS (MOL WT IN PARENTHESES) FOR MAIN HOMOLOGOUS SERIES MEMBERS OF ALIPHATIC ETHER LIPIDS OF *Monolepta australis*

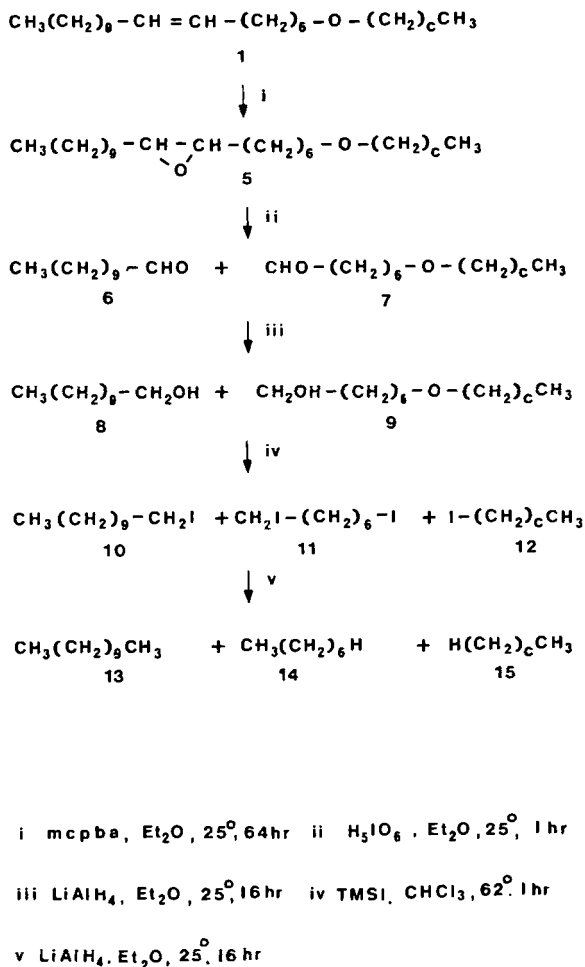
m/z	Peak No.				
	1	2	3	6	8
M+	10.7 (450)	4.4 (464)	6.5 (478)	< 1.0 (492)	5.4 (506)
281	5.3	5.2	3.3	10.8	6.8
250	40.6	10.5	16.2	19.5	25.4
M-267	9.9 (183)	< 1.0 (197)	6.6 (211)	6.3 (225)	5.4 (239)

chain was, as anticipated (Hallgren et al., 1959; Ryhage and Stenhagen, 1963; McCloskey and McClelland, 1965), not available from the mass spectral data. The M-267 and subsequent C_nH_{2n+1} fragments indicated that chromatographic peaks 1, 2, 3, 6, and 8 were, respectively, tridecyl ($c = 12$), tetradecyl ($c = 13$), pentadecyl ($c = 14$), hexadecyl ($c = 15$), and heptadecyl ($c = 16$) octadecenyl ether.

Of the numerous methods available for the location of double bonds in unsaturated fatty acid or other alkene derivatives (e.g., McCloskey and McClelland, 1965; Capella and Zorzut, 1968; Niehus and Ryhage, 1968; Abley et al., 1970; Dommès et al., 1976; Blomquist et al., 1980; Suzuki et al., 1981), a chemical degradation method best suited to monoenic series (Privett, 1966) was used. In our method (Scheme 2), the mixture of ethers (**1**) was epoxidized with 3-chloroperbenzoic acid, and the resulting epoxide series (**5**) was cleaved with periodic acid to give aldehydes **6** and **7**. Without separation, this mixture was reduced to the alcohols **8** and **9** and treated with the ether-cleaving agent trimethylsilyliodide (Jung and Lyster, 1977; Olah, 1979) to give iodides **10**, **11**, and **12**. These were reduced to their respective alkanes undecane **13**, heptane **14**, and a tridecane-heptadecane mixture (**15**) fixing the double bond in the 7-position of the octadecenyl chain in contrast with the 9-position as would be expected from an ether derived from the more usual oleyl derivatives. The retention times and indices of the major starting material and products are shown in Table 2. The saturated side of the ether linkage of the homologous series was confirmed by using GC to follow the presence of the five-membered homologous series through the entire reaction sequence (Scheme 2). In each reaction mixture, the presence of C_{13} to C_{17} alkyl chains in an approximate ratio of 13:3:60:2:3 was clearly observed, giving finally tridecane, tetradecane, pentadecane, hexadecane, and heptadecane (**15**). This then confirmed the structure of the homologous series as **1** where $a = 9$, $b = 6$, and $c = 12-16$, and GC peaks 1, 2, 3, 6, and 8 (Figure 1) were assigned, respectively, to tridecyl, tetradecyl, pentadecyl, hexadecyl, and heptadecyl 7-octadecenyl ether. Peaks 4, 5, 7, and 9 and their mass spectra are less definitive and await further identification.

CONCLUSION

This occurrence of long-chain, alkenyl-alkyl ethers in the lipid extract of *Monolepta australis* is, to our knowledge, the first reported occurrence of such compounds in either their natural or synthetic state. Recent reviews of insect lipids have not included any such ethers (Blomquist and Jackson, 1979; Hadley, 1981; Lockey, 1985; Lockey, 1986, personal communication). As far as we know, this then becomes the second formally reported occurrence of long-chain



SCHEME 2.

ethers in a biological environment. Although the isomeric 11-octadecenoxy radical is known in insects (vaccenyl acetate: Brieger and Butterworth, 1970; Meinwald et al., 1966; Jackson et al; 1981), the occurrence of 7-octadecenoxy derivatives in any biological environment is not known to us. On the other hand, the alkyl chain with predominantly odd numbers of carbon atoms is common and similar to half the *Locusta migratoria* lipid ethers, with carbon numbers C₁₃, C₁₅, and C₁₇ predominating (Genin et al., 1987).

Speculation about the taxonomic significance of the occurrence of these

TABLE 2. RETENTION TIMES (R_t) AND INDICES (R_i) FOR MAJOR STARTING MATERIAL AND PRODUCTS OF REACTION SEQUENCE SHOWN IN SCHEME 2 ($c = 14$)

	Compound											1:
	1	5	6	7	8	9	10	11	12	13	14	
R_t	20.13	21.47	2.63	14.09	3.27	14.61	5.08	5.55	16.15	1.20	4.95 ^a	4.
R_i	3322	3516	1290	2460	1364	2528	1526	1572	2735	1100	700	151

^aBPI 40-m column: 1 min at 40°C, 10°C/min to 250°C.

lipid ethers in insects as diverse as *M. australis* and *L. migratoria* would be premature until the lipid fractions of other insects have been investigated. Indeed, the advances in GC-MS technology that have facilitated the identification of these components may show that lipid ethers are widespread among insects. Speculation concerning biogenetic pathways is also awaiting the outcome of further investigation, although the presence of unsaturated chains with an even number of carbon atoms in *M. australis* and saturated chains with an even number of carbon atoms in *L. migratoria* indicates an extra step in the pathway of one species. Although our biological assays showed no pheromone activity for these ethers when tested on *M. australis*, their possible role in the physiology and/or behavior of *M. australis* also awaits the outcome of further investigation.

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(Z)-11-EICOSENYL ACETATE, AN AGGREGATION PHEROMONE IN *Drosophila malerkotliana*

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Abstract—(Z)-11-Eicosenyl acetate (Z11-20:Ac) was identified as the aggregation pheromone in *Drosophila malerkotliana*. The pheromone (200–300 ng/fly) was isolated from hexane extracts of the ejaculatory bulb of sexually mature male flies. Males released very little, if any, Z11-20:Ac to the food at any time. During mating there was a transfer of ca. 100 ng of Z11-20:Ac to the female's reproductive tract. The mated female fly transferred the Z11-20:Ac to the surrounding surfaces in just a few hours after mating. In bioassay in a wind-tunnel olfactometer, Z11-20:Ac was not attractive alone, but was synergistic with fermenting food or with acetone. Although *D. malerkotliana* has no (Z)-11-octadecenyl acetate (Z11-18:Ac), it was as attracted to Z11-18:Ac as to an equal quantity of Z11-20:Ac. *D. melanogaster* and *D. simulans*, however, responded to the Z11-18:Ac that they produced and did not respond to Z11-20:Ac.

Key Words—*Drosophila malerkotliana*, Diptera, Drosophilidae, aggregation pheromone, (Z)-11-eicosenyl acetate.

INTRODUCTION

Aggregation pheromones have been demonstrated in 11 species of *Drosophila*: *D. virilis* (Bartelt and Jackson, 1984; Bartelt et al., 1985a); *D. a. americana*, *D. a. texana*, *D. novamexicana*, and *D. lummei* (Bartelt et al., 1986); *D. borealis* and *D. littoralis* (Bartelt et al., 1988); *D. hydei* (Moats et al., 1987); *D. melanogaster* (Bartelt et al., 1985b), *D. simulans* (Schaner et al., 1987); and *D. mulleri* (Bartelt et al., 1989). In many of the closely related species, the

aggregation pheromones had the same or similar chemical structures, but between some species groups there appeared to be considerable differences in the chemical structures of the aggregation pheromones.

D. melanogaster and *D. simulans* of the *melanogaster* species group and *melanogaster* subgroup both used (*Z*)-11-octadecenyl acetate (Z11-18:Ac, *cis*-vaccenyl acetate, cVA) as their aggregation pheromone (Bartelt et al., 1985b; Schaner et al., 1987). No other fly-derived coattractant was necessary, but food or food-derived odors were synergistic with the Z11-18:Ac. In *D. melanogaster* and *D. simulans* the Z11-18:Ac was stored in the ejaculatory bulb of sexually mature male flies, transferred to females during mating, and released by mated female flies to the food media within hours after mating.

D. malerkotliana was chosen for aggregation pheromone investigation because it was in the *melanogaster* group but in the *ananassae* subgroup, and we could test whether male-derived compound(s) could function as an aggregation pheromone and whether the compound(s) were the same or differed from the Z11-18:Ac used by members of the *melanogaster* subgroup.

METHODS AND MATERIALS

Flies, Extraction, Chromatography, and Bioassay. *D. malerkotliana malerkotliana* (strain 14024-0391.0) was obtained from the National *Drosophila* Species Resource Center at Bowling Green, Ohio. It was originally collected at Mysore, India. *D. melanogaster* (wild type, Canton S strain) and *D. simulans* (strain 14021-0251.0) were as reported previously (Bartelt et al., 1985b; Schaner et al., 1987). Details of rearing, extraction, chromatography, and bioassay of the flies have been described previously (Bartelt and Jackson, 1984, Bartelt et al., 1985b; Schaner et al., 1987). Briefly, flies were separated by sex at 0-6 hr old and extracted at 6-7 days of age by soaking the flies in hexane at room temperature for 24 hr. Fractionation of the hexane extracts was on open columns of silicic acid eluted with hexane, 10% ether in hexane, 50% ether in hexane, and 10% methanol in methylene chloride. The 10% ether in hexane fraction was purified further by preparative gas chromatography (GC).

Bioassays were conducted in a wind-tunnel olfactometer containing ca. 1000 (0- to 2-day-old) flies that had been without food and water for approximately 2 hr. An extract, fraction, synthetic compound, or control solvent was applied to a filter paper strip inserted around the lip of the glass vial. Each bioassay test consisted of placing two differently treated vials, to be compared, into the olfactometer for 3 min. The bioassay data was transformed to the log ($x + 1$) scale before analysis to stabilize variance and analysis was done by the method of Yates' (1940).

Identification. Identification of the pheromone was done by comparing

retention times on GC, by gas chromatography-mass spectrometry (GC-MS) of the pheromone on a VG-MM16 GC-MS and by GC-MS of the dimethyl disulfide reaction product (Nichols et al., 1986). The determination of the isomer of the double-bond was done by comparison of synthetic Z11-20:Ac (Sigma Chemical, St. Louis, Missouri) and the fly-derived 11-20:Ac on a 30-m DB-1 capillary GC column with a temperature program of 140-220°C with a 4-min initial hold and a rate of 4°C/min. With this method the Z and E isomers of 9-18:Ac had previously been separated by a difference in the retention times of 0.11 min. The isomer of the fly-derived 11-20:Ac was determined by a coinjection of this compound with the synthetic Z11-20:Ac.

Production of Z11-20:Ac with Age. Since Z11-18:Ac was found in the ejaculatory bulb in *D. melanogaster* (Brieger and Butterworth, 1970), the ejaculatory bulbs of *D. malerkotliana* were removed, extracted with hexane, and analyzed by GC. To remove ejaculatory bulbs, the males were placed in the freezer for approximately 10 min and then placed, ventral side up, on a cork board with a dissecting pin through the thorax. The genital region was teased loose with forceps and placed in a drop of *Drosophila* Ringer's solution (Ephrussi and Beadle, 1936). Using two dissecting pins, the ejaculatory bulb was removed from the genital region under 45× magnification. Two sets of five bulbs for age groups from 2 hr to 8 days were placed in 1-ml conical vials that contained 500 ng nonadecane as an internal standard in 10 µl of hexane. The bulbs were smashed with the head of a dissecting pin and the hexane extract analyzed by GC. For comparison, we also analyzed hexane extracts of whole flies (3 × 20 flies) at each age. The flies were extracted at room temperature for 24 hr, and the hexane extracts were fractionated on open silicic acid columns described above. Z11-20:Ac was quantified by GC relative to the internal standard, Z11-16:Ac.

Transfer during Mating. The transfer of Z11-20:Ac from males to females and the subsequent release of the ester from the females was investigated. Virgin females (6-7 days old) were mated with virgin males of the same age. Immediately after completion of mating, the females were either extracted or placed into an empty 8-ml vial for 6 hr and then extracted. The vials were rinsed with 1 ml of hexane to recover any Z11-20:Ac that had been released. The mated males were treated in a similar fashion and, as controls, comparable sets of extracts were obtained from males and females that had not been allowed to mate. Each extract represented 10 fly equivalents and there were four replications per extract. The Z11-20:Ac in the extracts was quantitated by GC relative to an internal standard.

Female Reproductive Tract Removal. The female reproductive tract was also removed and the extract analyzed for the presence of Z11-20:Ac. Immediately upon completion of mating, the female was placed in the freezer for approximately 10 min. Viewed through a 10× dissecting scope, the female was

held ventral side up with forceps pressed laterally on the abdomen. The ovipositor was clasped with another pair of forceps and teased until the reproductive tract, without the ovaries, was pulled away from the fly. Two sets of five reproductive tracts were placed in conical vials with an internal standard and the extract analyzed as described previously for the ejaculatory bulbs. The hexane extract of female flies minus the reproductive tracts were analyzed by GC after they were soaked in hexane for 1 hr.

RESULTS AND DISCUSSION

Demonstration, Purification, and Identification of Pheromone. Initial attempts to demonstrate an aggregation pheromone in *D. malerkotliana* were patterned after previous experiments with *D. melanogaster* (Bartelt et al., 1985b) and *D. simulans* (Schaner et al., 1987). The hexane extract of mature males, with a food synergist, was clearly active in bioassay, and the hexane extract of mature females was, with food, also about twice as attractive as the food (Table 1, A). The male fly hexane fraction alone was not attractive. Although food was an effective coattractant, we felt that using a synergist that was less attractive might clarify pheromonal effects and make pheromone identification more rapid. Acetone was the preferred coattractant, which led to the identification of the aggregation pheromone in *D. melanogaster* (Bartelt et al., 1985b). Compounds such as acetone and ethyl acetate are known to be produced by molds and yeasts (Fogleman, 1982) and could well influence the behavior of the flies under natural conditions. When acetone was used as a coattractant in *D. malerkotliana* (Table 1, B), the male fly hexane extract plus acetone was about 10 times more attractive than the female fly hexane extract plus acetone. Acetone alone was not attractive. Both sexes of flies respond nearly equally to the hexane extracts of mature male flies. Since the attractive nature of the male fly extracts was much greater than that of female fly extracts, this paper will concentrate on the male fly-derived pheromone. After fractionation of the hexane extract of male flies on silicic acid, only the 10% ether-hexane fraction was active. The remaining fractions from the extract of male flies were not different in activity from the control solvent. By capillary GC, the male 10% ether-hexane fraction was predominantly one component. GC-MS of the major peak in the male 10% ether-hexane fraction indicated that the component was an acetate ester of a monounsaturated alcohol with 20 carbons. GC-MS of the dimethyl disulfide-treated fraction revealed that the double bond in the 20-carbon alcohol of the ester was at 11. Synthetic (Z)-11-eicosenyl acetate (abbreviated Z11-20:Ac) compared identically with the fly-derived component in GC and GC-MS. GC comparison of the fly-derived compound and the synthetic Z11-20:Ac identified the fly-derived 11-20:Ac as the Z isomer. Z11-20:Ac was present in the

TABLE 1. FOUR SERIES OF BIOASSAY EXPERIMENTS WITH EXTRACTS OF *D. malerkotliana* TO ISOLATE AND CHARACTERIZE AGGREGATION PHEROMONE.

Treatment ^a	Mean bioassay catch ^b
A: Synergism of food ^c by hexane extracts of flies	(N = 18)
Fermenting food	12.3d
Female fly hexane extract + food	26.8c
Male fly hexane extract + food	56.5b
Male fly hexane extract	0.7a
B: Comparison of hexane extracts from male and female flies with acetone as a coattractant	(N = 8)
Male fly hexane extract + acetone ^d	13.0b
Female fly hexane extract + acetone	1.2a
Acetone control	0.4a
C: Test for equivalence of synthetic Z11-20:Ac with extracts, fractions and purified compounds	(N = 16)
Synthetic Z11-20:Ac + acetone	11.0c
Male-derived Z11-20:Ac + acetone	6.1b
10% ether in hexane fraction + acetone	9.5bc
Male fly hexane extract + acetone	6.1b
Acetone control	1.0a
D: Synergism between Z11-20:Ac and fermenting food	(N = 24)
Synthetic Z11-20:Ac + food	73.3c
Fermenting food	5.3b
Z11-20:Ac	1.2a
Hexane control	0.7a

^aAll fly-derived fractions and their synthetic counterparts were used at 1 fly equivalent per test (220 ng of Z11-20:Ac).

^bIn any experiment, means followed by the same letter were not significantly different at the 5% level (LSD).

^cFermenting food is Formula 4-24 Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, North Carolina) to which active yeast has been added at least 24 hr previous to testing.

^dAcetone, 10 μ l, was added to the filter paper with the extracts.

hexane extract of mature male flies at a concentration of ca. 200 ng/fly, and there was no indication of the presence of Z11-18:Ac in the extract. This is the first report of Z11-20:Ac as an aggregation pheromone in *Drosophila*.

The male hexane extract, 10% ether-hexane fraction, synthetic Z11-20:Ac, and male-derived Z11-20:Ac were compared by bioassay (all at 220 ng Z11-20:Ac; Table 1, C). All were significantly greater than the control ($P < 0.001$). In this study, the synthetic Z11-20:Ac was significantly more attractive than the male hexane extract and male-derived Z11-20:Ac ($P < 0.05$). The 10% ether-hexane fraction was intermediate in attraction. However, Z11-20:Ac accounts for all the activity of the male hexane extract.

Synergism of Z11-20:Ac with food. Since Z11-18:Ac was synergistic with food in other *Drosophila* species (Bartelt et al., 1985b; Schaner, et al., 1987), the synthetic Z11-20:Ac was tested for synergism with food. In this bioassay there was a 14-fold synergism of synthetic Z11-20:Ac with food (Table 1, D).

Response of Flies to Z11-Acetates of 16, 18, and 20 Carbons. When synthetic Z11-20:Ac, Z11-18:Ac, and Z11-16:Ac along with acetone were compared in bioassay (Table 2), *D. malerkotliana* not only responded strongly to Z11-20:Ac but also to Z11-18:Ac, the aggregation pheromone of *D. melanogaster* and *D. simulans* (Bartelt et al., 1985b; Schaner et al., 1987). No attraction was detected toward Z11-16:Ac. In contrast, *D. simulans* and *D. melanogaster* were strongly attracted only to their own aggregation pheromone. *D. melanogaster* also preferred Z11-16:Ac significantly over controls. A similar study was conducted with the *virilis* group (Bartelt and Jackson, 1984; Bartelt et al., 1986; 1988), where both the number of carbons in the alkene pheromone, as well as the position and stereochemistry of the double-bond were investigated. In *D. virilis* the aggregation pheromone was (Z)-10-heneicosene, but in *D. a. americana*, *D. a. texana*, and *D. novamexicana* it was (Z)-9-heneicosene. *D. lummei*, *D. borealis*, and *D. littoralis* possessed no heneicosenes, but curiously all three responded well to (Z)-9-heneicosene, and *D. lummei* and *D. littoralis* responded to (Z)-10-heneicosene. Therefore, it is not uncommon for a species to be attracted to the aggregation pheromone of a closely related species even though it does not possess it.

TABLE 2. ESTER SPECIFICITY^a FOR *D. malerkotliana*, *D. melanogaster*, AND *D. simulans*

Ester ^b	Bioassay flies (N = 18) ^c		
	Mal	Mel	Sim
Z11-16:Ac	4a	23b	9a
Z11-18:Ac	157b	100c	100b
Z11-20:Ac	100b	6a	-8a

^aEster specificity is described by an index of activity: $I = (\text{test ester} - \text{control}) / (\text{standard ester} - \text{control}) \times 100$, where the three means were taken from a balanced incomplete block experiment, the "standard ester" is the male-specific ester for that species. The index compares esters on a percent scale, corrected for controls.

^bEsters used at 800 ng for *D. melanogaster* (mel) and *D. simulans* (Sim) and 220 ng for *D. malerkotliana* (Mal) (~1 fly eq), 10 μ l of acetone was used as coattractant.

^cWithin each column, indices of activity followed by the same letter were not significantly different at the 0.05 level and those followed by the letter "a" were not significantly different from the control. Comparisons of means between columns are not meaningful because the experiments were conducted on different days with different species of flies.

Dose Response to Z11-20:Ac. Increasing amounts of Z11-20:Ac lead to greater relative aggregation activity but the dose response was essentially log-linear (Figure 1, $r = 0.9503$, $P < 0.05$). The dose of 200 ng is approximately the hexane extractable amount from the mature male fly, so 200 ng was given the relative value of 100. A 20-ng dose was only 2.8% as attractive as the 200-ng dose and may be near the minimum quantity of Z11-20:Ac that these flies can detect. Between 200 ng and 2000 ng there was a nearly twofold increase in response. The increase in response to 20,000 ng of Z11-20:Ac was slightly over twofold and showed no sign of reaching a plateau.

Location, Production, and Transfer of Z11-20:Ac. The ejaculatory bulbs from mature (6 days old) male *D. malerkotliana* were analyzed and found to contain approximately 270 ng/fly of Z11-20:Ac. The rest of the fly contained 22 ± 5 ng/fly of Z11-20:Ac, so most of the aggregation pheromone is located in the ejaculatory bulb of mature male flies. During the first 24 hr after eclosion the male flies have very little Z11-20:Ac, during the next 48 hr there is a rapid

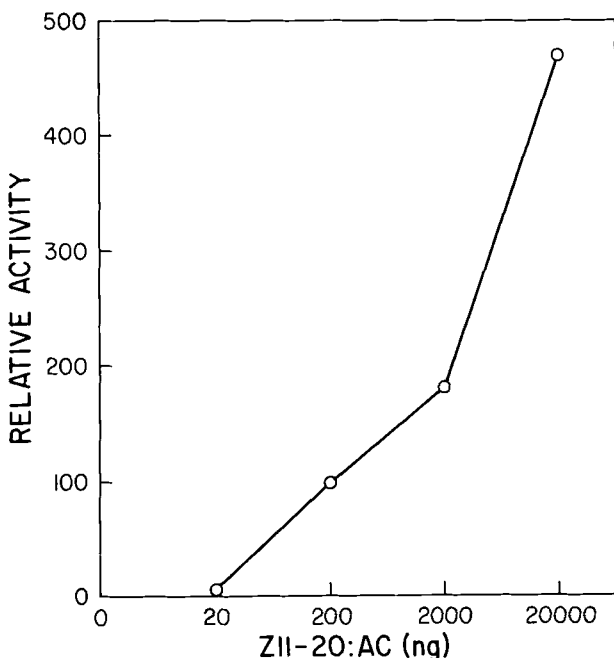


FIG. 1. Activity of *D. malerkotliana* towards different doses of Z11-20:Ac. Relative activities are 100% for the 200-ng dose of Z11-20:Ac (~ 1 fly eq) and 0% for the controls ($N = 8$). In each case, 10 μ l of acetone was used as a co-attractant. Spacing along the x axis is logarithmic.

increase in Z11-20:Ac. Over the next few days there is a gradual increase to a plateau of about 300 ng/fly (Figure 2). The assays of ejaculatory bulb contents are somewhat higher than the 24-hr hexane extracts of whole flies. The same trend in Z11-20:Ac production is observed from the whole fly and ejaculatory bulb extracts. Although the whole fly extracts are much easier to perform, they do not provide a reliable quantitative estimation.

Virgin females possess no Z11-20:Ac. However, males transferred over half of the Z11-20:Ac they possessed to the female during mating. Immediately after mating about 85% of the male-transferred Z11-20:Ac is in the female's reproductive tract. Within 6 hr after mating, the female transferred approximately one third of the Z11-20:Ac into an empty vial. Males, virgin or mated, emitted no Z11-20:Ac into an empty vial that they occupied for 6 hr. Therefore, as in *D. melanogaster* and *D. simulans* (Bartelt et al., 1985b; Schaner et al., 1987), it is the mated female that emits the pheromone into the environment although it is male-produced.

In summary, sexually mature *D. malerkotliana* males produce Z11-20:Ac,

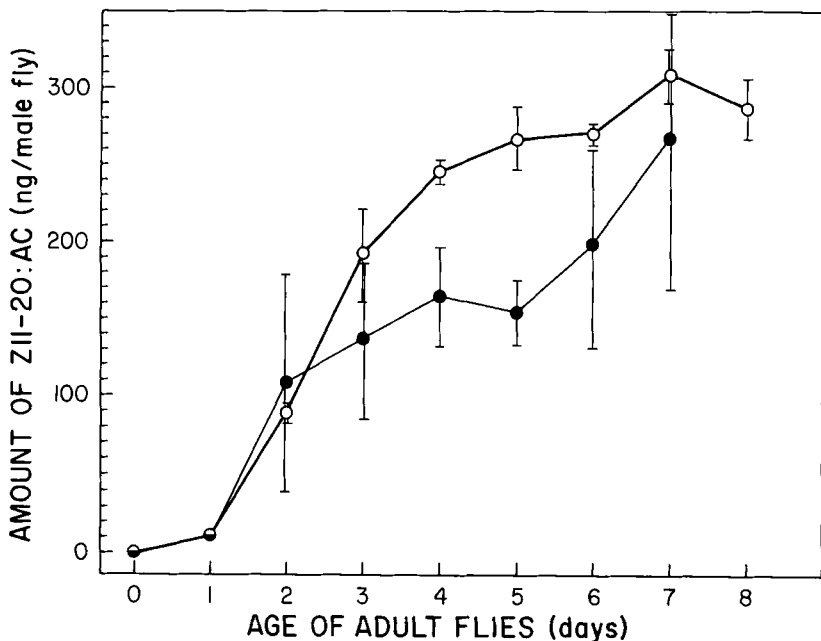


FIG. 2. Amounts of Z11-20:Ac extracted from virgin males *D. malerkotliana* of various ages. Solid circles indicate the Z11-20:Ac from a 24-hr hexane soak of the whole fly, and open circles indicate the Z11-20:Ac within the hexane extract of ejaculatory bulbs. The bars indicate standard deviation.

which is stored in the ejaculatory bulb, transferred to the reproductive tract of females during mating, and released from the females to the surrounding surfaces after mating. Along with food odors, Z11-20:Ac acts as an aggregation pheromone to attract *D. malerkotliana* of either sex to food and potential mating sites.

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PHENOLIC BIOSYNTHESIS, LEAF DAMAGE, AND INSECT HERBIVORY IN BIRCH (*Betula pendula*)

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Abstract—The effect of both caterpillar herbivory and artificial damage on phenylalanine ammonia lyase (PAL) activity of birch foliage was measured, using an intact cell assay. After artificial damage there was a small increase in PAL activity in damaged leaves but no change in adjacent undamaged ones. Insect grazing produced a larger increase in PAL activity, and the enzyme activity was also increased in adjacent undamaged leaves. Artificial damage increased the phenolic levels of the damaged leaves. Insect grazing caused a larger, longer-lasting increase in phenolic levels and also elevated phenolic levels in undamaged leaves. The possible role of these wound-induced biochemical changes in birch is discussed.

Key Words—Phenolics, PAL activation, insect herbivory, plant resistance, *Betula pendula*, *Apocheima pilosaria*, Lepidoptera, geometridae.

INTRODUCTION

Wound-induced reductions in leaf palatability to insect herbivores have been reported in *Betula pubescens* Ehrh. (Haukioja and Niemela, 1977) and *Betula pendula* Roth (Wratten et al., 1984). Such changes can occur in damaged and adjacent undamaged leaves, and the responses may be part of a wound-induced antiherbivore defense mechanism (Haukioja and Hahnimaki, 1985; Bergelson et al., 1986; Lawton, 1987). It has been suggested that the decrease in palatability is a result of an increase in the concentration of phenolic compounds known to occur in damaged leaves (Niemela et al., 1979; Wratten et al., 1984; Bergelson et al., 1986). However, palatability to insect herbivores does not always correspond well with phenolic levels (Hartley and Lawton, 1987).

Changes in the phenolic metabolism of damaged birch leaves were investigated at a more fundamental level than has hitherto been possible. Simple phenolic compounds are derived from the shikimate pathway (Conn, 1981). Consequently, by studying the activity of enzymes in this pathway in damaged and undamaged leaves, it may be possible to define, in more precise biochemical terms, the way in which plants respond to insect attack. Differences in the chemical changes induced by artificial damage as opposed to insect-grazing can thus be characterized in terms of enzyme activity, which could be important in assessing whether the changes are likely to be involved in defense against herbivores or pathogens, or simply in repair of wounded tissues. For example, an increase in phenolic compounds in undamaged leaves due to increased enzyme activity in these undamaged tissues, rather than due to transport of phenolics from the site of damage, may indicate a mechanism which is not purely tissue repair.

In the present study, the activity of phenylalanine ammonia lyase (PAL) was investigated, together with the concentration of phenolic compounds, in damaged and undamaged leaves from both insect-grazed and artificially damaged trees. Evidence is presented that PAL activity is stimulated by insect attack on birch, even in undamaged leaves, and the response differs from that caused by artificial damage to the leaves.

METHODS AND MATERIALS

PAL Measurements. On July 8, 1986, 12 birch trees, each about 2 m high, were chosen in the University of York tree nursery, where they had been transplanted several years earlier. Twenty-five *Apocheima pilosaria* larvae (Lepidoptera, Geometridae) were caged on three branches on each of four of the trees and allowed to graze overnight, by which time 10–15% of the leaf area of the caged branches had been removed. A further four trees had the same amount and distribution of damage inflicted on three of their branches with a pair of scissors (sterilized in alcohol to reduce the risk of fungal infection). The four remaining trees were left as controls. Fifteen leaves were collected from each tree at the start of the experiment, and from the control trees one, three, five, and seven days later, while 15 damaged, and 15 undamaged leaves immediately adjacent to damaged ones, were removed from the treatment trees on the same days. The undamaged leaves included some at the same node, some above, and some below the damaged leaves, as available.

Enzyme activity was measured immediately after the leaves were collected using a modified version of the intact cell assay of Amrhein et al. (1976). This "in vivo" method for assaying PAL was chosen in preference to an in vitro enzyme assay in order to obviate possible differences in enzyme recovery from the damaged and adjacent leaves.

One half gram (wet weight) of 6-mm leaf disks was incubated with 5 μCi L-phenyl(2,3- ^3H)alanine (Amersham; specific activity 43 Ci/mmol) in 2 ml of 0.15 M potassium phosphate buffer (pH 5.5) for 3 hr. The labeled phenylalanine was absorbed by the cells, equilibrated with the endogenous substrate, and the action of PAL liberated labeled N^3H_2 . This lost ^3H to tissue water to produce ^3HOH , a virtually irreversible process due to the considerable dilution of the tracer, so recycling is very unlikely.

The ^3HOH was recovered from the medium by sublimation by a procedure similar to that of Mitra et al. (1975). Twenty microliters of incubation medium, together with a drop of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, was placed on a filter paper disk that was positioned on a pin stuck into the lid of a scintillation vial. Vials were placed in a piece of polystyrene such that the bottom of the vial was in liquid nitrogen while the top was heated with an infrared lamp until the CoCl_2 solution turned blue. The pin and filter paper were then removed, and the ^3H activity of the ice formed was determined using a scintillation counter (LKB), following the addition of 2 ml of Optiphase MP (Fisons) to the vial. The PAL activity was measured as counts per minute (cpm) per 0.5 g fresh weight per 3 hr, after correction for background and control counts. (Control counts are the values recorded from incubations using boiled leaves with no PAL activity.) The results were expressed as mean percentage changes over the activity at the start of the experiment (to reduce the effect of the between-tree variation in initial PAL activities) and were analyzed in a one-way ANOVA, tested separately each day. The specific treatments responsible for producing significant effects in the ANOVA were identified using nominated comparison *t* tests.

The leaf material remaining after the enzyme assay had been carried out was freeze-dried and assayed for total phenolics as described below.

Field Experiment. On May 15, 1985, at Skipwith Common, 10 miles south of York (grid ref. SE 664379), 15 small *Betula pendula* trees (each about 1.5 m high) were selected, sprayed with insecticide (Sprayday, Pan Britannica Industries Ltd.; contains resmethrin and pyrethrum), and all natural damage marked with a small dot of paint on leaf petioles. Three weeks later, the leaves of five of the trees were left undamaged, five were cut with scissors, and five were grazed by *Apocheima pilosaria*, as in the experiment described above.

Twenty leaves were collected from each of the control trees at the start of the experiment, and one, three, five, and eight days subsequently. In the case of the other treatments, 20 leaves were collected before damage, but on days one, three, five, and eight, 20 undamaged leaves from a branch adjacent to a treatment branch were collected, in addition to the 20 damaged leaves. Leaves were placed on ice immediately after collection, returned to the laboratory within 1 hr, and stored at -20°C .

Chemical Analyses. All chemical analysis (except for PAL activity) was carried out on freeze-dried material. Total phenolics were measured using the Folin-Denis method as described in Bergelson et al. (1986), except that 20 mg

of leaf powder was extracted and after centrifugation the volume was adjusted to 10 ml, and then aliquots equivalent to 0.1 and 0.2 mg of leaf-powder were used in the analysis. Protein precipitation was measured using the Bradford method (Bradford, 1976). The procedure was similar to that of Martin and Martin (1982), but slightly modified as described in Hartley and Lawton (1987). The protein-precipitating properties were expressed as the coefficients (slopes) of regression lines fitted to the graphs of milligrams (dry weight) of leaf extracted vs. grams of BSA precipitated (see Martin and Martin 1982 for further details).

RESULTS

PAL activity was measured in control leaves and in damaged and adjacent undamaged leaves from insect and artificially damaged trees (Figure 1). The activity was found to vary somewhat on a daily basis (Figure 1), with large between-tree differences; hence, although there was a significant effect of treatment on activity on both days 1 ($F = 10.64$, $df = 4, 15$, $P = 0.0003$) and 5 ($F = 5.46$, $df = 4, 14$, $P = 0.0084$), there were no significant effects on day 3, or on day 7, by which time activities in all treatments had decreased to near control levels. In the artificially damaged trees, cut leaves had significantly higher PAL activities than leaves from the control trees one day after damage

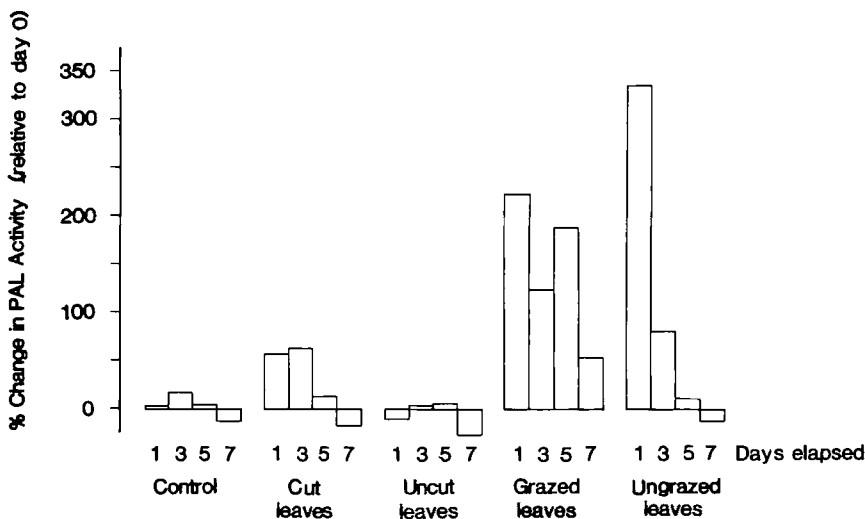


FIG. 1. The percentage change in PAL activity in damaged and adjacent undamaged birch leaves over the seven days subsequent to damage, and in control trees. Each value is the mean of measurements of four trees (the same four trees for each sample).

($t = 4.03$, $df = 6$, $P < 0.01$), but by day 5 the activity had declined, and there was no longer a significant difference. At no time did the undamaged leaves from these trees show a significant increase in their PAL activity. On the insect-grazed trees, PAL activities were greatly increased, with the damaged leaves having higher activities than both control and cut leaves ($t = 6.64$, $df = 6$, $P < 0.001$, and $t = 5.09$, $df = 6$, $P < 0.02$, respectively). On these trees, the adjacent undamaged leaves had the largest increase in activity of all on day 1 ($t = 3.39$, $df = 3.2$; $P < 0.05$), but this was short-lived; by day 3 there was no significant difference from the controls. However, the elevation of PAL activity in the insect-damaged leaves was more persistent and, unlike the artificially damaged leaves, the level of activity five days after damage was still significantly higher than in control trees ($t = 2.58$, $df = 6$, $P < 0.05$).

Samples of the leaves used for enzyme assays were analyzed for total phenolics, and the phenolic levels were found to be elevated in the leaves with increased PAL activity (Figure 2). However, since the amount of phenolics accumulating depends on factors such as the rate of phenolic degradation (which was not measured), and because of the imprecise method used to estimate phenolics, PAL activity and phenolic levels do not correspond exactly. The control leaves and the undamaged leaves on cut trees showed no significant changes in phenolic content, but the cut leaves, and both the damaged and undamaged leaves on the insect-grazed trees all had increased amounts of phenolic compounds (paired t tests, $t = 6.64$, $df = 3$, $P < 0.01$; $t = 13.6$, $df = 3$, $P < 0.001$; and $t = 5.79$; $df = 3$; $P < 0.02$, respectively). Insect attack produced a larger increase in phenolics than artificial damage (two sample t test, $t = 3.52$, $df = 6$, $P < 0.02$).

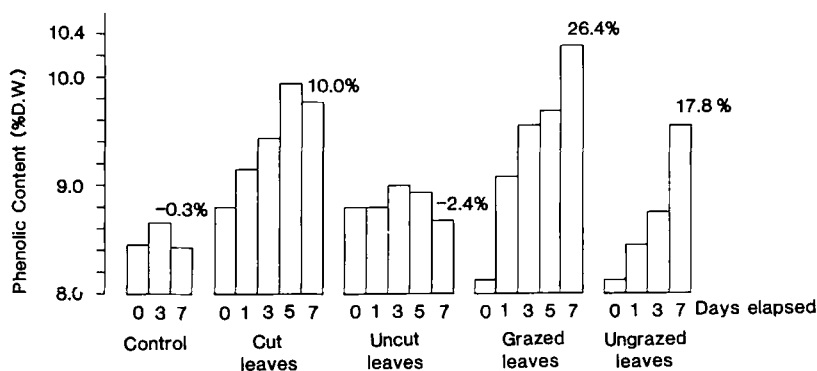


FIG. 2. The effect of leaf damage on phenolic levels in birch leaves. The values are the means of measurements made on four trees, and the same trees were used on each sampling day. The percentage change in phenolic content after seven days is also shown.

The results of the 1985 field experiment also show chemical differences between insect and artificially damaged leaves. The level of phenolic compounds in control trees (Figure 3) did not differ significantly over an eight-day experimental period (paired *t* test, $t = 1.36$, $df = 4$; $P > 0.1$). In trees with leaves that were cut with scissors (Figure 3), the phenolic levels increased in the damaged leaves (paired *t* test, $t = 5.91$, $df = 4$, $P < 0.01$), but not in undamaged ones from an adjacent branch (paired *t* test, $t = 1.33$, $df = 4$, $P > 0.1$). Again, in insect-grazed leaves, the phenolics rose to higher levels than in cut leaves (two sample *t* test on eight-day values: $t = 3.14$, $df = 8$, $P < 0.02$), and undamaged leaves on the neighboring branch to the grazing also had slightly increased phenolic levels after eight days (paired *t* test, $t = 2.99$; $df = 4$, $P < 0.05$). However, the change in the undamaged foliage was less than in the 1986 experiment, perhaps because the undamaged leaves in that experiment were immediately adjacent to the insect-damaged ones.

The protein-precipitating abilities of extracts of artificially damaged, insect-grazed, and control trees were also made. However, these measurements failed to reveal any significant increases in protein-precipitating ability resulting from insect grazing, although a slight, but statistically significant, increase was recorded in artificially damaged leaves (data not shown). These results suggested that assays of protein-precipitation ability and Folin-Denis measurements were not equivalent as indicators of insect-induced chemical changes, possibly because they were measures of different kinds of chemicals, and no further protein precipitation measurements were made.

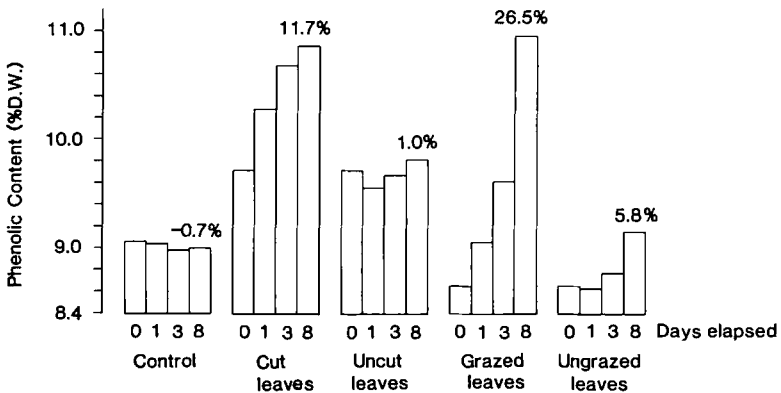


FIG. 3. The effect of leaf damage on the phenolic content of birch leaves. Each value is the mean of measurements of five trees, each tree sampled on the days shown. The percentage change in phenolic content after eight days is also shown.

DISCUSSION

It is clear from previous work (e.g., Wratten et al., 1984), and from the results presented here, that phenolic concentrations are elevated in insect-grazed birch leaves and are also higher in undamaged leaves adjacent to those which were damaged. The finding that PAL activity is also increased in such leaves suggests that these elevated levels of phenolic compounds are the consequence of increased *in situ* biosynthetic activity, rather than mobilization of phenolics from the site of damage. The response to artificial damage was different, however, with smaller changes both in terms of PAL activity and phenolic concentration, and no changes occurring in adjacent undamaged leaves. It has been suggested that such a response, in which insect damage is a more effective inducer of chemical changes than artificial damage and in which synthesis of phenolics occurs at sites remote from damage, represents an active and specific defense, rather than a wound-repair mechanism (e.g., Haukioja and Neuvonen, 1985). However, a number of questions require further investigation before the role of these wound-induced changes is clear.

First, is the increased PAL activity the result of enzyme activation or enzyme synthesis? A number of stimuli, such as light, chemicals, and fungal infection, can increase PAL activity in plants, and it has been shown in some cases that increased synthesis of mRNA coding for PAL is associated with this increase in PAL activity (e.g., Dixon, 1986).

The second question is, what is the nature of the transmitted factor that passes from insect-grazed leaves to adjacent leaves, resulting in elevated PAL activity? Transmittable factors have been reported in plants following damage (Ryan, 1974), and, interestingly, "elicitors" capable of inducing PAL in plant cells are found in some fungal-plant interactions (Hahlbrock et al., 1981).

The fact that increased PAL activity is a common response of plants subject to fungal attack raises a third question: what is the relevance of the increased PAL activity found in the insect-grazed leaves? Although insect grazing had a more marked effect on PAL activity than an equivalent amount of damage inflicted with scissors, this could simply reflect the contamination of caterpillar mouthparts with fungal spores (Shain and Hillis, 1972). A second possibility is that grazing produces a more severe stress on the leaf by a sustained period of physical damage.

The function of the damage-induced increase in phenolic levels is also hard to assess since the responses of leaves to damage might include the induction of many chemicals, some of which may be primarily antifungal and some of which might play a role in defense against insects. Phenolics have certainly been associated with resistance to fungal attack (Werder and Kern, 1985), as well as antiherbivore effects (Bennett, 1965; Todd et al., 1971; Roehrig and

Capinera, 1983), as has leaf damage (Karban et al., 1987). However, the role of phenolics is not entirely clear, as both insects (Bernays, 1981) and fungi (Shaw, 1985) can remain unaffected, and birch-feeding insects often seem indifferent to the chemical changes induced in damaged leaves (Lawton, 1987; Hartley and Lawton, 1987; but see Bergelson et al., 1986; Wratten et al., 1984).

Although the ecological significance of the reported changes in PAL activity remains to be established, this study has clearly shown a differential response of birch trees to insect and artificial damage and that insect attack produces marked changes in phenolic biosynthesis in both grazed and adjacent undamaged leaves. Furthermore, these results suggest that the *in vivo* PAL assay may prove to be a sensitive measure of insect and mechanical damage-induced chemical changes. Further studies are needed on other plant species and on the mechanism of increasing PAL activity to progress towards a better understanding of the way in which plants detect and respond to insect attack.

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STRUCTURE-ACTIVITY RELATIONSHIP OF FLAVONES AS GROWTH INHIBITORS OF THE NAVEL ORANGEWORM

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Abstract—A series of flavones with widely varying degrees of substitution was fed to neonate larvae of the navel orangeworm. Growth of navel orangeworm larvae is inhibited by 5-methoxy flavones and flavone itself; 5-hydroxy flavones do not inhibit growth. Host resistance of citrus fruit to attack by the navel orangeworm might be due to the 5-methoxy flavones that occur in the peels.

Key Words—*Amyelois transitella*, Lepidoptera, Pyralidae, citrus, flavones, growth inhibition, host-plant resistance, navel orangeworm, nobiletin, orange, tangeretin.

INTRODUCTION

The navel orangeworm (NOW), *Amyelois transitella* (Walker), was first described as a pest in the Salt River Valley of Arizona in 1922 where it was found on navel oranges (Mote, 1922). By 1925 the NOW had been found on grapefruit, lemons, many varieties of oranges, figs, pears, and rotting pomegranates. Although fear of spread to the California citrus groves was prevalent, scientific studies indicated that the worm was a secondary pest, occurring mainly on split or damaged fruit (Wade, 1961). It is unlikely that the peels of citrus fruit act as a physical barrier to the NOW since the larvae have mandibles strong enough to bore through nut shells (Michelbacher and Davis, 1961). It is more likely that chemical constituents in the peels act as feeding deterrents or growth inhibitors making the fruit unattractive to NOW larvae.

The peels of citrus fruits are typically rich in pectins, essential oils, caro-

tenoid pigments and flavones, especially flavanone glycosides and highly methoxylated flavone aglycones (Horowitz and Gentili, 1977). Flavones (Figure 1) exhibit a wide range of biological activities on insects, including growth inhibition (Chan et al., 1978; Elliger et al., 1980). The three fully methoxylated flavones, nobiletin (Table 1, 4) tangeretin (Table 1, 6), and heptamethoxyflavone (Table 1, 3), all occur in the peels of tangerines, oranges, and grapefruit. The corresponding 5-hydroxy analogs (Table 1, 10–12) are found in tangerines and oranges (Tatum and Berry, 1972). These six citrus flavones and 14 additional flavones with a wide variety of substitution patterns were tested in an artificial diet for growth inhibiting properties on NOW larvae. The resulting relationship between structure and growth inhibition of NOW larvae is presented here.

METHODS AND MATERIALS

Flavones 1 and 7 (Table 1) were purchased (Sigma Chemical Co., St. Louis, Missouri) and recrystallized. Flavones 13–20 were available from earlier studies of the American desert shrub *Gutierrezia microcephala* (Roitman and James, 1985). Flavones 3, 4, and 6 were isolated from tangerine peel by extraction, solvent partition, and repetitive silicic acid chromatography. Flavones 2 and 9 were kindly provided by Dr. L. Jurd of these laboratories. Flavone 8 was prepared from benzoyl chloride and 2',6'-dihydroxyacetophenone, and flavone 5 was prepared by methylation of 8 with dimethyl sulfate in acetone solution in the presence of potassium carbonate. Flavones 10 and 11 were prepared by demethylation (ethanolic hydrochloric acid) of tangeretin (6) and nobiletin (4), respectively. Flavone 12 was prepared by partial methylation (methyl iodide, acetone, potassium carbonate, reflux 3 hr) of flavone 19. All flavones were chromatographically homogeneous, and their physical and spectroscopic properties agreed well with values reported in the literature.

For each concentration of flavone tested (1, 2, 4, and 8 mmol/kg wet diet),

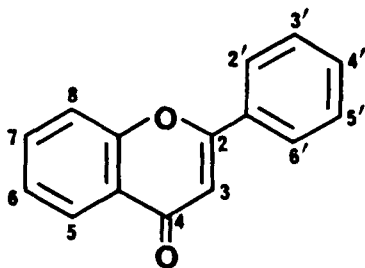


FIG. 1. The flavone skeleton.

TABLE 1. FLAVONES TESTED FOR GROWTH INHIBITION

	Substitution		Common name	ED ₅₀ ^a	
	Hydroxylation	Methoxylation		mmol/kg	ppm
1	—	—	Flavone	0.4	89
2	—	3,5,7,3',4'		0.9	330
3 ^b	—	3,5,6,7,8,3',4'	Heptamethoxyflavone	1.1	480
4 ^b	—	5,6,7,8,3',4'	Nobiletin	1.1	440
5	—	5		1.7	430
6 ^b	—	5,6,7,8,4'	Tangeretin	1.9	710
7	3,5,7,3',4'	—	Quercetin	NA	
8	5	—	Primuletin	NA	
9	5	3,7,3',4'		NA	
10 ^b	5	6,7,8,4'	5-O-Desmethyltangeretin	NA	
11 ^b	5	6,7,8,3',4'	5-O-Desmethylnobiletin	NA	
12 ^b	5	3,6,7,8,3',4'		NA	
13	5,7	3,6,8,3',4',5'		NA	
14	5,7,4'	3,6,8		NA	
15	5,7,4'	3,8,3'		NA	
16	5,7,4'	3,6,8,3'		NA	
17	5,7,4'	3,6,8,3',5'		NA	
18	5,7,3',4'	3,8		NA	
19	5,7,3',4'	3,6,8		NA	
20	5,7,8,3',4'	3		NA	

^aConcentration at which larval growth is inhibited 50%.

^bFound in citrus peel.

^cNot active, ED₅₀ greater than 8 mmol flavone/kg diet.

two identical 6-g portions of artificial diet were prepared as described below. Scarcity of material precluded preparation of duplicate diets for flavones 15, 17, 18, and 20. An acetone or methanol solution of the flavone to be tested was stirred into 50 mg of alpha-cellulose. After the solvent had evaporated, 4.6 g of codling moth diet (Bioserve) was added and mixed to homogeneity with 1.4 ml of a 2% aqueous solution of agar. Control diets were prepared in an identical manner including alpha-cellulose but no flavone. Each diet was allowed to solidify, then cut into 10 equal pieces, each of which was placed in a 2-ml disposable conical cup (Markson Science). Two neonate larvae were then placed in each cup. After 14 days at 26°C (day length 14 hr), the largest larva in each cup was weighed, providing a total of 2 × 10 larval weights for each concentration of each flavone. For each flavone concentration a mean larval weight was calculated and compared to the control mean larval weight to give the percent growth inhibition. For flavones that showed growth inhibiting activity, four

additional concentrations bracketing the ED_{50} (the concentration at which larval growth is inhibited by 50%) were tested to confirm its value. Standard deviations of 15–20% from the mean weight were noted for larvae of control weight size; larger standard deviations were noted for the smaller weights of the stunted larvae. Flavones whose ED_{50} was greater than 8 mmol/kg wet weight diet were considered inactive.

RESULTS AND DISCUSSION

Flavones with either no substitution or methoxy substitution at the 5-position are active as growth inhibitors of NOW larvae (Figure 2 and Table 1), whereas flavones with hydroxy substitution at the 5-position are inactive. Since a 5-hydroxy substituent is strongly hydrogen bonded to the 4-carbonyl oxygen, growth inhibition is correlated with the availability of this carbonyl. Flavone (1), with no substituents to form chemical or steric interactions with the 4-carbonyl, proved to be the most potent growth inhibitor of the flavones tested. The presence of a single hydroxy substituent at the 5 position (compound 8) completely destroys growth inhibition, whereas 5-methoxyflavone (5) remains active as a growth inhibitor, albeit less active than flavone.

Quercetin (7) is active as a growth inhibitor for several insects (Chan et al., 1978), but is inactive against the NOW, as expected due to its 5-hydroxy

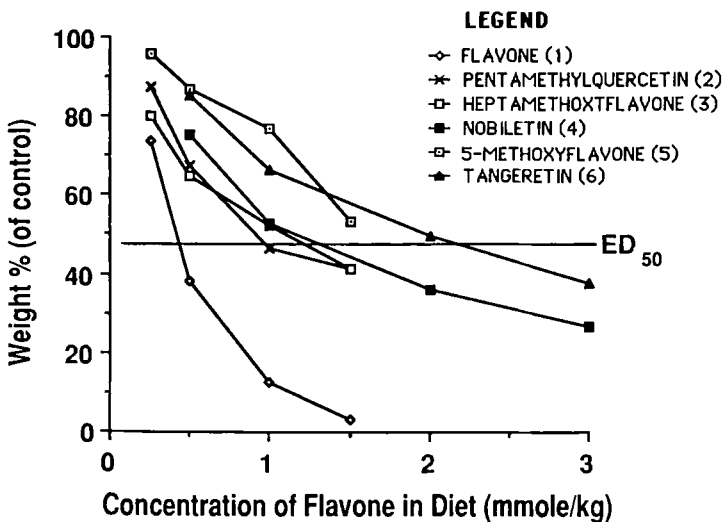


FIG. 2. Activity of flavones on navel orangeworm.

substitution. Fully methoxylated quercetin (2) is active, whereas the analog with 5-hydroxy substitution (9) is inactive. The same pattern is observed for the citrus flavones. The permethoxylated flavones nobiletin (4), tangeretin (6), and heptamethoxyflavone (3) all show growth-inhibiting activity, whereas their 5-hydroxy analogs (11, 10, and 12, respectively) are inactive. Other 5-hydroxy flavones (13–20) exhibiting diverse hydroxy and methoxy substitution patterns all proved to be inactive as growth inhibitors.

Although this structure–activity relationship does not shed light upon the mechanism of growth inhibition, it is consistent with the theory that secondary plant products are factors in host-plant resistance. Flavones with a 5-methoxy substitution, to which the NOW is sensitive, are relatively rare in nature; permethoxylated flavones are seldom found outside the citrus family. For example, of the 11 flavones found in orange peel, nine are fully methoxylated and only two have a free 5-hydroxyl. The predominant orange peel permethoxylated flavones are nobiletin (4) and sinensetin (5,6,7,3',4'-pentamethoxyflavone), with moderate amounts of tangeretin (6), heptamethoxyflavone (3), tetra-*O*-methylscutellarein (5,6,7,4'-tetramethoxyflavone), and 3,5,6,7,3',4'-hexamethoxyflavone; in addition small amounts of 5,7,8,4'-tetramethoxyflavone, 3,5,7,8,3',4'-hexamethoxyflavone, and 5,7,8,3',4'-pentamethoxyflavone have been found (Tatum and Berry, 1972). As all of these flavones contain a 5-methoxy group, it may be assumed they would be active as growth inhibitors of NOW. Nobiletin, which has an ED_{50} of 440 ppm, has been found in orange peel at levels of 190 ppm (Park et al., 1983) to 800 ppm (Sastry et al., 1964). Orange juice, on the other hand, contains nobiletin at approximately 1 ppm, with the five major permethoxylated flavones present at a total of 4–6 ppm (Veldhuis et al., 1970). It appears that these permethoxylated flavones play a major role in protecting undamaged oranges from NOW attack. The larvae do not penetrate the peel, which contains flavone levels high enough to cause significant growth inhibition, but rather gain access through splits or fungally damaged spots in the peel and then eat the pulp, which contains very much lower levels of the permethoxylated flavones.

Despite its name, the NOW is currently not a citrus pest, having found more suitable hosts, especially walnuts and almonds. The chemical defense provided by the citrus peel appears to be a factor preventing the NOW from becoming a more prominent pest in citrus. Compounds such as the permethoxylated flavones, which play a role in the host-plant resistance of citrus to the NOW, may be useful in protecting more vulnerable crops. The techniques of genetic engineering may allow development of walnut and almond husks containing protective levels of 5-methoxy flavones.

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SCENT LURES FROM ANAL SAC SECRETIONS OF THE FERRET *Mustela furo* L.

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Abstract—A bioassay of eight synthetic anal sac compounds showed that ferrets were most attracted to a mixture of 2-propylthietane and *trans*- and *cis*-2,3-dimethylthietane. This mixture was used as the basis of an artificial scent lure. Trapping experiments used two traps at each site. In comparing trap success, ferrets chose artificial lure in preference to no odor, and chose both food baits and natural-product anal sac odor over artificial lure. However, the effectiveness of artificial lure was demonstrated by comparison between trap sites with bait-lure pairs and those with lure only. The comparison indicated that scent lures should be as effective as bait in attracting ferrets, and this was confirmed in a field program. We conclude that scent lures are valuable additions to the current techniques used in mustelid control operations, but more work is needed to increase the inherent species specificity of scent lures for mustelids and to develop a simple means of making a lure that will release odor for a long time.

Key Words—Ferret, *Mustela furo*, lure, scent, thietane, sulfur-containing compounds, anal sac secretion, wildlife management, trapping.

INTRODUCTION

Introduced mustelid species (ferrets, *Mustela furo*; stoats, *M. erminea*; weasels, *M. nivalis*) have had a significant impact as predators of native fauna in New Zealand. Native birds in Kowhai Bush, Kaikoura, lost 67% of their nests to predators, with stoats and weasels being responsible for 78% of this predation

(Moors, 1983, and personal communication). Predation has been the main reason for nesting failure of the endangered black stilt (*Himantopus novaeseelandiae*), with ferrets being one of the main predators (Pierce, 1982). Merton (1978) noted that indigenous bird life has been depleted on New Zealand offshore islands inhabited by introduced mustelids. Predation by stoats cannot be excluded from consideration of factors limiting the population of takahe, *Notornis mantelli* (Lavers and Mills, 1978; King, 1984) and North Island kokako, *Callaeas cinerea wilsoni* (King, 1984). Another mustelid problem occurs at Tairoa Head, Otago, where some royal albatross chicks, *Diomedea ephemphora*, may fall prey to ferrets (King and Moors, 1979). This predation has led to a need for effective damage control procedures that require the exploration of new mustelid control measures.

Ferrets use anal sac odors as a means of communicating sexual and individual identity (Clapperton et al., 1988). This knowledge, and the fact that various components of ferret anal sac secretions have been identified and can be synthesized (Crump, 1980a), suggested that compounds from anal sac secretions could be used as the active ingredients of ferret trap lures.

The use of synthetic scents to lure animals to control devices has the potential advantages of increasing the effectiveness, efficiency, and species specificity of a control program. These features are also of value when trapping is part of ecological research rather than a control program. In addition, inedible scent lures are advantageous in studies where the stomach contents or faeces of the captured animals are to be examined and edible bait can be confused with prey. Synthetic scent lures also have consistent chemical properties, an important feature if research is aimed at estimating population densities by monitoring numbers of visits to scent stations.

In this study we investigate the responses of ferrets to components of their anal sac secretions in a laboratory bioassay experiment, and we report field tests of the efficacy and species specificity of scent lures containing the active components.

PART I. LABORATORY BIOASSAY

Methods and Materials

We conducted four bioassay experiments, using a T maze (Clapperton et al., 1988), to determine the relative attractiveness of the components of ferret anal sac secretions. The terms "attractive" and "attractiveness" are used to describe the investigative responses of ferrets and do not imply any subjective like or dislike of the odors. Measures of attractiveness included speed of approach to the odor source, and time spent sniffing at the odor source. The compounds are shown in Figure 1 and the various combinations of compounds

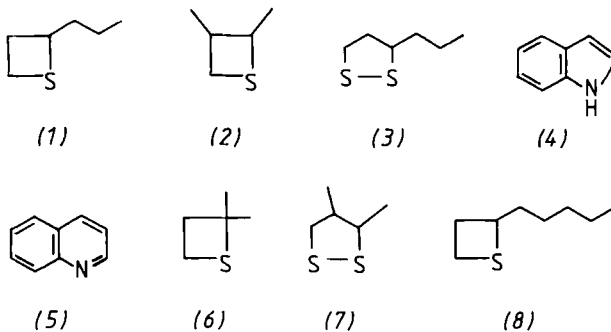


FIG. 1. The eight chemical compounds used in the experiments. (1) 2-propylthietane, (2) 2,3-dimethylthietane, (3) 3-propyl-1,2-dithiolane, (4) indole, (5) quinoline, (6) 2,2-dimethylthietane, (7) 3,4-dimethyl-1,2-dithiolane, and (8) 2-pentylthietane.

tested in each experiment are listed in Table 1. The thietane and dithiolane compounds were synthesized according to Crump (1980a). The indole and quinoline were commercial products. All these pure synthetic compounds were diluted with hexane to a ratio of one part odor compound to five parts hexane. For the combinations of two odor compounds the ratio was 1:1:10. These synthetic odor solutions were kept in 1-ml Teflon-sealed glass vials, and stored at -10°C .

All trials were conducted between 8 AM and 5 PM. Only three trials were run on any one day, and each animal was tested no more than once every four days. The subject was placed in the start-box 5 min before the beginning of a trial. During this time $5\ \mu\text{l}$ of the odor solution was mixed with two drops (ca. $50\ \mu\text{l}$) of diethylene glycol succinate (DEGS), to reduce the rate of evaporation, and spread on a piece of filterpaper. This odor sample was then placed in one arm of the maze, and a blank (filterpaper plus DEGS and hexane) in the other arm. Thirty seconds elapsed before the door was raised and timing began. For each 15-min trial we recorded: (1) the animal's initial choice of odor port, (2) the time the subject took to approach within 10 cm of the odor port, and (3) the

TABLE 1. COMBINATIONS OF SYNTHETIC COMPOUNDS USED IN BIOASSAY EXPERIMENTS

Experiment	Combinations of compounds ^a					
I	1	2	3	1 + 2	1 + 3	2 + 3
II	1 + 2	4	5	1 + 2 + 4	1 + 2 + 5	Blank
III	1 + 2	6	7	1 + 2 + 6	1 + 2 + 7	6 + 7
IV	1 + 2	8	1 + 2 + 8			

^aCompounds numbered as in Figure 1.

total time spent sniffing the odor and the blank. At the end of the trial the animal was removed and the apparatus cleaned using chlorine bleach and water. At least 4 hr separated one trial from the next, allowing time for the apparatus to dry completely.

The anal sac compounds used in the four experiments (Table 1) were chosen to determine the most attractive combinations of compounds. The combination 1 + 2, which was most attractive in experiment I, was used throughout the remaining experiments. This combination served as a comparison for the attractiveness of other compounds and was also used in the thought that it might, in combination with other compounds, form the basis of an even more attractive mixture.

Each subject was tested against all the odor combinations. Experiments I–III were Latin-square designs, balanced to avoid residual effects of order of presentation of the odors. Odor/blank positions were randomized to offset right–left preferences in the maze. In experiments II and III the subjects more often investigated the right odor port first (36 right vs. 11 left in experiment II, 38 right vs. 19 left in experiment III). This did not occur in experiments I and IV (28 right vs. 28 left, 17 right vs. 11 left, respectively). In experiment IV only three odor combinations were tested.

The time data were ranked and then normalized before analysis. Analysis of variance was used to test the effects on response time of the sex of the subjects, the odor that they were tested against, and the order in which the odors were presented. Some animals in the experiments had been bred in captivity and were therefore younger and tamer than the wild-caught individuals. In each of the four experiments, we used two males and two to five females that were captive-bred. A single dummy covariate (0 or 1) was used to account for the possible effect of tameness and/or age. Some animals did not run the maze, and their responses were treated as missing data. Missing data were estimated in the analysis of variance and degrees of freedom adjusted appropriately (Rothamsted Experimental Station, 1980). The Tukey test was used to detect significant differences between levels of a factor.

Results

Experiment I. There were significant differences between the times spent sniffing at the various odors in experiment I ($F_{5,35} = 7.3$, $P < 0.0005$; Figure 2a). Males and females did not respond differently, nor was the interaction between odor and sex a significant factor, but the covariate for tameness explained a significant amount of the variation in the subjects' responses ($F_{1,7} = 13.8$, $P < 0.01$). The combination of compounds 1 and 2 was the most attractive overall and to both males and females separately. It was significantly more attractive than all the other odors except for compound 1 on its own,

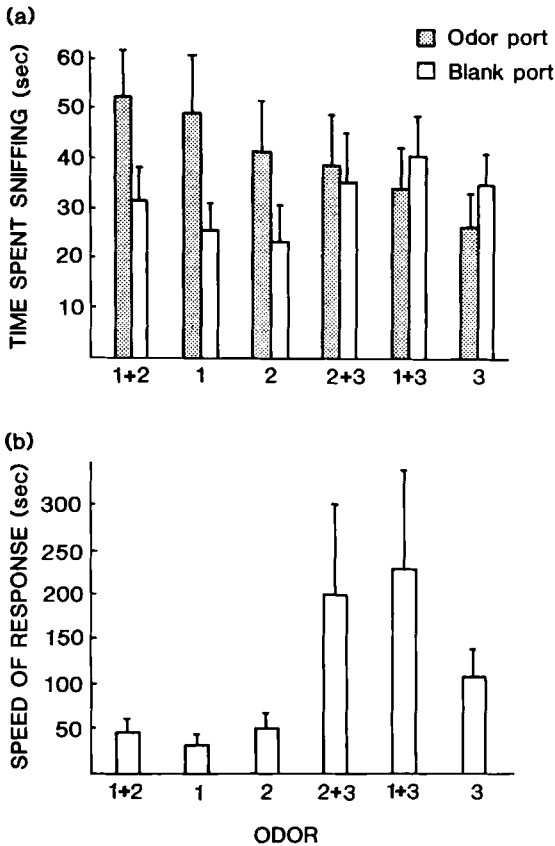


FIG. 2. Subjects' responses to odors in bioassay experiment I. Data are untransformed means and standard errors ($N = 10$).

which was significantly different only from compound 3 ($P < 0.005$, Tukey; Figure 2a). In comparisons of the time spent investigating the odor port and the blank port, the ferrets spent significantly longer at the odor port with compounds 1, 2, and 1 + 2 ($P < 0.01$, $P < 0.05$, $P < 0.014$, respectively, Wilcoxon).

There were also significant differences between the subjects' speed of approach to the various odors ($F_{5,35} = 4.2$, $P < 0.005$; Figure 2b). Males and females did not, overall, show differences in speed of responses, even when the differences in tameness and/or age were accounted for, but they did respond differently to different odors ($F_{5,35} = 4.8$, $P < 0.0025$). Overall, it was again compound 1 + 2 that provided the most attractive odor, although the speed of response to this combination differed significantly only from that of compound

3 ($P < 0.025$, Tukey) and 1 + 3 ($P < 0.05$, Tukey). Males and females differed in that males responded relatively faster to compound 3 and slower to 2 + 3 than did females. The order of presentation did not affect the relative attractiveness of the odors.

Experiment II. No simple pattern of responses emerged in experiment II. There was a significant difference in the time spent sniffing the different odors ($F_{5,25} = 6.9$, $P < 0.005$), with 1 + 2 and 4 being significantly more attractive than either no odor ($P < 0.01$, Tukey) or 1 + 2 + 4 ($P < 0.025$, Tukey; Figure 3a). Males and females found different odors more attractive ($F_{5,25} = 6.6$, $P < 0.001$). For females, the addition of 5 to 1 + 2 reduced its attractiveness to less than that of no odor (but not significantly so) and 5 on its own

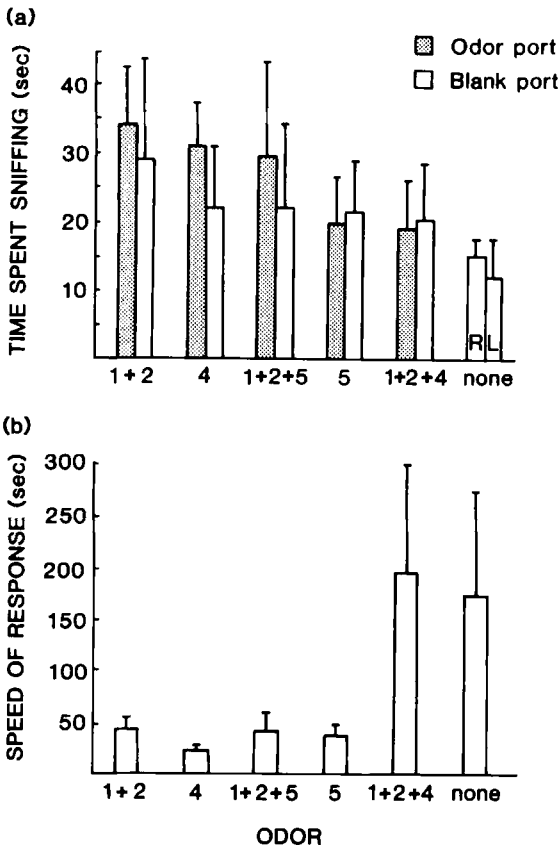


FIG. 3. Subjects' responses to odors in bioassay experiment II. Data are untransformed means and standard errors ($N = 8$). R = right odor port, L = left odor port.

was less attractive still. In males, 1 + 2 + 4 was no more attractive than no odor, and these two were significantly less attractive than 4, 1 + 2, and 5 ($P < 0.01$, $P < 0.01$, $P < 0.025$, Tukey, respectively). There was no difference in the time spent at the odor and blank ports for any of the odors tested (Figure 3a).

In terms of speed of response, females responded significantly faster than males ($F_{1,5} = 11.3$, $P < 0.025$), and there was no significant effect of tameness and/or age of the subjects. Overall, there were significant differences in the subjects' speeds of response to the various odors ($F_{5,25} = 4.7$, $P < 0.005$), due mainly to a rapid response to 4 compared to 1 + 2 + 4 ($P < 0.01$, Tukey) and the blank ($P < 0.025$, Tukey, Figure 3b). Males and females responded at different speeds depending upon the odor being tested ($F_{5,25} = 2.7$, $P < 0.05$), with the males responding relatively faster to 5 than the females did. Order of odor presentation was not an important factor determining either the time spent sniffing the odors or speed of response.

Experiment III. In experiment III the subjects spent significantly different lengths of time sniffing the different odors ($F_{5,35} = 3.2$, $P < 0.025$). The sexes did not differ significantly, nor was there a significant sex-odor interaction, but the young tame animals spent significantly longer at the odors than did the adults ($F_{1,7} = 14.0$, $P < 0.01$). The most attractive odor was again 1 + 2, but this was significantly greater than compound 6 only ($P < 0.025$, Tukey, Figure 4a). The ferrets showed no preferences for sniffing longer at the odor or the blank ports (Figure 4a), nor were there any significant differences in the speeds of response (Figure 4b). Order of presentation had no effect.

Experiment IV. In experiment IV no significant differences in attractiveness were found, either in the time spent sniffing the odors, or in speed of response (Figure 5a,b). There was a tendency for the subjects to be less attracted to compound 8 than compound 1 + 2. The subjects spent longer sniffing at the odor port with 1 + 2 compared to the blank ($P < 0.01$, Wilcoxon), but showed no preferences for odor and/or blank ports when the test odor was 8 or 1 + 2 + 8 (Figure 5a).

PART 2. FIELD TRIALS OF SCENT LURES

Methods and Materials

Live-Trapping Experiment. We carried out trapping experiments at Puke-puke Lagoon Wildlife Management Reserve, Manawatu, New Zealand, between February 1984 and June 1985, to test wild ferrets' preferences for various trap lures and baits. We used 42 Edgar live-traps (King and Edgar, 1977), 650 mm long \times 150 mm wide \times 160 mm high. They were given one coat of polyurethane varnish inside for ease of cleaning and removing odors. Synthetic lures

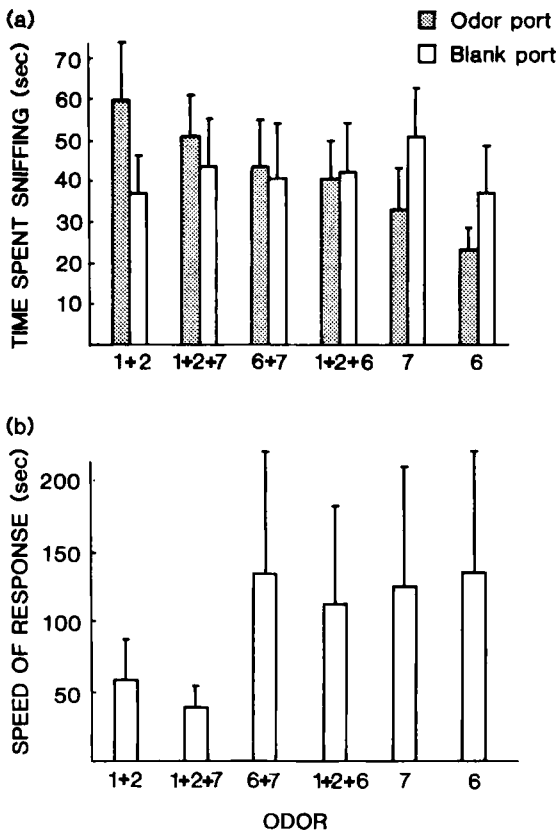


FIG. 4. Subjects' responses to odors in bioassay experiment III. Data are untransformed means and standard errors ($N = 10$).

consisted of one part odor compound to five parts hexane or pentane by volume. The diluted odor compounds were then incorporated in white petroleum jelly which made up 93% by weight of the lure. The odors tested as lures were: lure A was half 2-propylthietane and half *trans*- and *cis*-2,3-dimethylthietane; lure B contained only 2-propylthietane; lure C only indole; and lure D was a "natural" lure prepared from female anal sac extracts mixed with white petroleum jelly to produce a lure of approximately the same strength as the synthetic lures. The blank control lure consisted of 93% white petroleum jelly and 7% hexane. A smelly natural bait was made from dead laboratory mice cut open.

In the late afternoon, bait was placed directly into the traps and lures were smeared onto 40 × 40-mm galvanized iron plates, which were then put in the traps. Care was taken to avoid contaminating the next trap with the previous

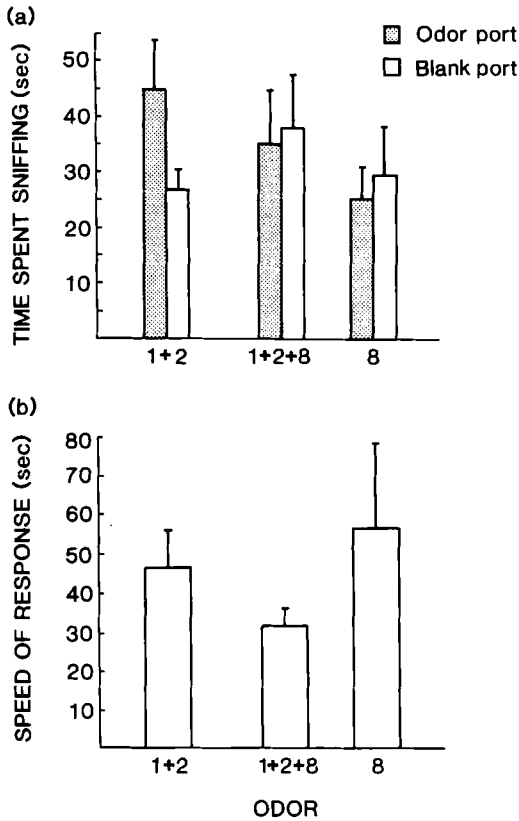


FIG. 5. Subjects' responses to odors in bioassay experiment IV. Data are untransformed means and standard errors ($N = 9$).

treatment. The traps were checked the following morning, cleaned with water and chlorine bleach, and closed. Prior to release at the same site, captured ferrets were ear-tagged, sexed, and their breeding condition noted. The trap position and treatment were also recorded. Other animals caught in the traps were identified and released.

In a preliminary trial in February 1984, 40 traps were set out singly throughout the reserve. The traps were sited on animal runways to maximize the likelihood of catching ferrets at every site. The traps were operated for eight consecutive nights. On each of the first four nights one quarter of the traps had each of the following treatments: bait plus lure A, bait only, lure A only, and clean. The traps were divided into groups of four, and treatments assigned

according to a Latin-square design. At the end of four nights all the traps were moved to new sites and the experiment repeated.

In all subsequent trials 21 trap sites were used. At each site two traps containing different treatments were set side by side. Trap sites were not changed within a trial. A trial consisted of six consecutive nights of trapping. The trap sites were divided into groups of three and the treatment pairs rotated among them. Each pair of treatments had equal exposure at each site, and the experimental design was balanced for order of presentation. From March to May 1984 treatments were renewed every day, while in subsequent trials treatments were renewed every second day, with the treatments being swapped between the traps at each site for the second night. A trap site was closed for one night if it had caught a ferret the night before. The data were analyzed using chi-square goodness-of-fit and independence tests to determine the effect on capture success of treatment, sex of the captured ferrets, time of year, freshness of treatment, and independence of these factors.

Kill-Trapping Experiment. The synthetic lure A was incorporated into the regular predator trapping program of the New Zealand Wildlife Service in the Mackenzie Basin, South Canterbury, from September 4, to October 10, 1984. Ninety-one leghold gin traps were set singly in an irregular pattern. Bait (fresh rabbit or occasionally hare or fish) was hung on a hook and lure put on a metal plate behind the trap in "blind" sets, and the plate camouflaged to prevent removal by magpies (*Gymnorhina tibicen*) and to avoid direct sun. In "walk-through" sets, the bait or lure plate was hidden underneath the trigger platform. Traps were checked and reset each morning or early in the afternoon.

Traps were treated alternately with lure and with bait. Treatments were renewed every third day and swapped between traps every third or sixth day to counterbalance location bias. The variation in numbers of animals caught relating to treatment, freshness of treatment, and species and sex of captured animals were analyzed using chi-square goodness-of-fit and independence tests.

Results

Live-Trapping Experiments. In the preliminary live-trapping experiment at Pukepuke Lagoon in February 1984, 25 ferrets were caught over the eight nights, six on bait plus lure traps, nine on bait only traps, seven on lure only, and three on clean traps. These differences in success rates are not significant.

The paired-trap design experiments yielded capture rates of 4–20 ferrets per six nights of trapping, with the same individuals being regularly caught.

Over the 325 trap nights in which bait vs. lure A trap pairs were operated, significantly more ferrets were caught on bait than on lure A ($P < 0.005$), whereas there was no significant difference between the choice of treatment in the breeding and nonbreeding seasons or between the sexes. The first-night

treatments were not significantly more successful than second-night treatments (for bait $\chi^2_1 = 0.17$, for lure $\chi^2_1 = 2.15$), and choice of treatment was independent of freshness of treatment (Table 2).

The difference in the numbers of captures on bait and lure D were not significant (Table 2). However, when comparing these results with bait vs. lure A, the trend to catching more ferrets on bait than on lure was independent of the type of lure ($\chi^2_1 = 1.60$). Only females were caught on lure D, giving a significant difference between the male and female choices ($P < 0.05$). Captures on these two treatments did not vary with season or with freshness of treatment (Table 2).

Lure D was preferred over lure A ($P < 0.05$), and choice was independent of season, sex, and freshness (Table 2). Significantly more ferrets were caught on lure A than on the blank treatment ($P < 0.025$), and again males and females had similar likelihoods of being caught on the two treatments. All four ferrets captured on the blank treatment were trapped on the second night of treatment,

TABLE 2. NUMBER OF FERRETS CAUGHT ON VARIOUS TREATMENTS IN LIVE-TRAPPING EXPERIMENTS

Treatment pairs	Total	Season		Male	Female	Night	
		Nonbreeding	Breeding			1st	2nd
Bait vs. Lure I	17 4	6 2	11 2	5 1	12 3	12 4	5 0
χ^2_1	8.05***	0.22, NS		0.02, NS		1.62, NS	
Bait vs. Lure D	16 9	9 6	7 3	6 0	10 9	8 6	8 3
χ^2_1	1.96, NS	0.28, NS		4.36*		0.68, NS	
Lure A vs. Lure D	4 13	0 4	4 9	1 5	3 7 + 1?	1 9	3 4
χ^2_1	4.76*	0.35, NS		0.40, NS		2.58, NS	
Lure A vs. Blank	14 4	5 1	9 3	5 3	7 + 2? 1	11 0	3 4
χ^2_1	5.55**	0.14, NS		1.42, NS		7.90**	
Lure A vs. Lure B	13 9	11 6	2 3	6 3	7 5 + 1?	8 5	5 4
χ^2_1	0.73, NS	0.93, NS		0.12, NS		0.07, NS	
Lure A vs. Lure C	14 10	7 4	7 6	6 2	7 + 1? 8	5 5	9 5
χ^2_1	0.67, NS	0.22, NS		1.65, NS		0.51, NS	

NS = not significant, * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$. ? = sex unknown. Statistical comparisons are chi-square goodness-of-fit and independence tests with correction factor for small ($N < 10$) expected values (Pirie and Hamden, 1972).

giving a significant dependence between choice and freshness of treatment ($P < 0.025$). Lure A was preferred over the blank in both the nonbreeding and breeding seasons. Both lure B and lure C were as attractive as lure A, and in neither case was the success of the different lures dependent upon season, sex, or freshness of treatment (Table 2).

Comparisons of the captures among treatment pairs reveal that those trap-sites having two lure traps were just as successful as those with one bait and one lure trap ($\chi_1^2 = 0.06$, NS), and trap site success was independent of sex ($\chi_1^2 = 2.7$, NS; Table 3). Overall, trap site success was also independent of season ($\chi_1^2 = 0.64$), but males were caught more often at sites with two lures in the breeding season than expected by chance ($\chi_1^2 = 4.6$, $P < 0.05$; Table 3).

Seven nontarget species were caught (Table 4). The most common was the hedgehog (*Erinaceus europaeus*), which was caught in traps independent of treatment type. Seven weasels were caught, all but one on scent lures, and the two stoats caught were both on scent lures. Five Norway rats (*Rattus norvegicus*) were caught on the artificial lures, and none on the natural product lure (lure D).

Kill-Trapping Experiment. In the Mackenzie Basin study area gin traps treated with artificial scent lure (Lure A) were as successful in trapping ferrets as those baited with meat (Table 5). The catch rate per 100 trap nights on lure was 2.11, compared to 1.79 on bait.

More males than females were caught on lure, due to an overall higher catch of males (41 males to 24 females), but treatment success was independent of sex (Table 5). The number of ferrets caught on lure varied with freshness of treatment, with most ferrets being caught when the lure was fresh, and only five being caught on 3-day-old scent ($P < 0.025$). The catch rate on bait did

TABLE 3. NUMBER OF CAPTURES ON BAIT VS. LURE A AND BAIT VS. LURE D COMBINED (501 TRAP NIGHTS), COMPARED TO THOSE ON ALL THE LURE VS. LURE AND LURE A VS. BLANK PAIRS (878 TRAP NIGHTS)

Treatment	Season			
	Breeding		Nonbreeding	
	Male	Female	Male	Female
Bait vs. lure	4	18	8	16
Lure vs. lure	19	23	12	22

TABLE 4. NUMBER OF CAPTURES OF NONTARGET SPECIES DURING VARIOUS TREATMENTS OF LIVE-TRAPPING EXPERIMENTS

Treatments	Nontarget species						
	Weasel	Stoat	Rat	Hedgehog	Possum	Cat	Mouse
Bait vs.	1			4			
Lure A	3			8	1		
Bait vs.			1	2		1	
Lure D	1			7			
Lure A vs.			1	5			
Lure D				7			
Lure A vs.			3	9			1
Blank			1	5	4		
Lure A vs.	1	1		4			
Lure B	1			4	2		
Lure A vs.				6			
Lure C		1	1	9	1		

not drop significantly over the rights, but the rate of drop in catch rate was independent of treatment (Table 5).

The captures of important nontarget species are summarized in Table 6. Sixteen cats (*Felix catus*) were caught, only three of them on lure ($P < 0.025$),

TABLE 5. COMPARISON OF NUMBER OF FERRET CAPTURES ON TWO TREATMENTS, BAIT (1727 TRAP NIGHTS) AND ARTIFICIAL SCENT LURE (1605 TRAP NIGHTS) IN KILL-TRAPPING EXPERIMENT

	Bait	Lure
Total	31	34
χ^2_1		0.44, NS
Male	18	23
Female	13	11
$\chi^2_{1, 2 \times 2}$		0.64, NS
1st night	11	19
2nd night	12	10
3rd night	8	5
χ^2_1	0.84, NS	6.67, $P < 0.025$
$\chi^2_{2, 2 \times 3}$		2.64, NS

TABLE 6. COMPARISONS OF NUMBER OF CAPTURES OF NONTARGET SPECIES ON TWO TREATMENTS, BAIT AND ARTIFICIAL SCENT LURE, IN KILL-TRAPPING EXPERIMENT

Species	Bait	Lure
Cat	13	3
	$\chi^2_1 = 5.55, P < 0.025$	
Harrier	29	0
	$\chi^2_1 = 26.95, P < 0.001$	
Hedgehog	22	14
	$\chi^2_1 = 1.24, NS$	
Stoat	0	2

while none of the 29 harriers (*Circus approximans*) caught were on lure ($P < 0.001$). Hedgehogs were caught equally often on bait and lure (1.27 and 0.87 per 100 trap nights, respectively). Only two other mustelids were caught, both stoats, and both on lure.

DISCUSSION

This study has shown that ferrets find some components of their anal sac odors more attractive than others and that these odorous compounds could be used as scent lures to attract ferrets to traps.

Bioassay Experiments. In all four bioassay experiments it was the combination 2-propylthietane (compound 1) and 2,3-dimethylthietane (compound 2) that was the most attractive, based on time spent investigating the odors. The results of the experiments based on speed of response are not so clear, but in all experiments the odor of 1 + 2 did not elicit a significantly slower response than any of the other odors. Compounds 1 and 2 were the most volatile of the compounds tested and are usually present at high concentrations in female anal sac extracts; compound 1 is also an important component of male odors (Clapperton et al., 1988).

In experiment II, indole (compound 4) proved to be very attractive. Indole is the least volatile of the compounds tested. The combination of 1 + 2 + 4 was not as attractive as either 1 + 2 or 4 alone. Perhaps the fact that 2 is often strong in female odors and 4 is strong in male odors (Clapperton et al., 1988) means that their combination at equal concentrations produced a confusing signal to the subjects. Similarly, combining compounds 5, 6, or 8, all of which are normally minor components of ferret anal sac odors (Crump, 1980a; Clapperton et al., 1988), with 1 + 2 at equal concentrations may not have been an appropriate way of testing these combinations.

Trapping Experiments. Results of both the long-term live-trapping and the intensive kill-trapping experiments demonstrate the viability of scent lures for attracting both male and female ferrets to control devices. Although ferrets preferred to enter bait traps when given a choice, trap sites with lure alone were just as successful as those with bait in both experiments. It could be argued that the lack of differences in trap site success indicates that ferrets would have been caught if no odor was present. However, previous workers have found mustelid traps to be more successful when they contain some odor, either bait or the smell of a previous capture, than when there is no odor present (King, 1973; Lavers, 1973; King and Edgar, 1977). A nonsignificant trend for lower success of clean traps was also seen in the preliminary trial of this study. This would imply that it was the treatments, not some other feature of the traps (e.g., simply their location), that accounted for their success in capturing ferrets. This is also indicated by the significant drop in catch rate on scent lure in the kill-trapping experiment (and a similar trend on bait) as the treatments became stale.

Experimental Design. The difference in results between the no-choice trapping experiments (one trap per site) and choice trapping experiments (two traps per site) has implications for the design of experiments for testing the effects of odors on trappability. If only choice experiments had been used, these may have led to the incorrect conclusion that scent lures would not be as effective as bait in attracting ferrets. Recent investigations on rodents have used choice experiments, with two or three traps with different treatments at each site, to look at the effects of different odors on trappability and, ultimately, their effects on population estimates (Daly et al., 1980; Stoddart, 1980; Wuensch, 1982; Stoddart and Smith, 1984). However, the demonstration of a choice does not imply that, given only one trap to enter, odors will be sufficient to attract or deter animals from entry. Given sufficient replication and an experimental design that does not allow the siting of traps to be a confounding factor, such information is obtainable from no-choice experiments (e.g., Roughton and Bowden, 1979; Gorman, 1984; Van der Berk and Müller-Schwarze, 1984). Choice experiments can, however, give information on the complex social interactions found in communities of small mammals (Stoddart and Smith, 1984). An experimental design that includes analysis of both within- and between-trap-site differences in trap success rate provides more information than either approach on its own (e.g., Stoddart, 1982; this study).

Species Specificity. None of the scent lures were totally species-specific. The capture of the other mustelids on the artificial ferret scent lures is not surprising, considering that one of the components, 2-propylthietane, is an important component of stoat anal sac secretions (Crump, 1980b) and that weasel anal sac secretions contain 2,3-dimethylthietane (Brink et al., 1983). Cats were not attracted by the lures, and harriers were not expected to be caught on the lure as they are visual rather than olfactory hunters (Brown, 1976). Hedgehogs

were caught on all treatments and are thus either indiscriminate about odor or else do not use olfactory cues to determine trap entry. Rodents were not caught on the natural product lure, but did enter traps containing the synthetic lures.

Experimental Limitations. The difficulty of demonstrating significant differences in responses when sample sizes are small is evident in both the laboratory experiments and field trials. This is a common problem in bioassay experiments on mammals (e.g., Müller-Schwarze et al., 1974; Singer et al., 1976; Murphy et al., 1978; McKenna Kruse and Howard, 1983; Crump et al., 1984), and is "inevitable in field experiments with carnivores" (King, 1980).

The repeated use of the same individuals introduces the possibility of the animals habituating to the odor stimuli. Repeated exposure to the odor of 1 + 2 in the bioassay experiments may have led to the ferrets showing relatively high responses to the "novel" odors in the later experiments. This tendency for ferrets to investigate novel stimuli has been demonstrated by Hughes (1964). Even so, 1 + 2 was always as attractive as the other odors tested. In the live-trapping experiments the same individuals were regularly trapped, but the fact that significant differences occurred between captures on some pairs of treatments suggests that ferrets were not becoming "trap-happy," but were making choices based on odor cues.

Future Direction. To be of more use in mustelid control programs or ecological research, improvements to the artificial scent lures should include a determination of the most effective concentration of odor compounds. This would require knowledge of the distance over which ferrets can perceive odors, and their responses to odors of varying concentration. The formulation of a slow-release scent lure should also be investigated. Such a formulation could prove the true advantage of lure over bait by providing a long-lasting attractant that can be used in an extensive trapping program, thereby overcoming the relatively rapid decay in the efficacy of both lure and bait demonstrated in this study. Another future direction of work should be to investigate whether components of ferret anal sac secretions repel other animals, e.g., rodents. The inclusion of such repellent compounds in a lure also containing ferret attractant might maintain the efficacy of a ferret scent lure and at the same time increase its species specificity.

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MYRISTICIN, SAFROLE, AND FAGARAMIDE AS PHYTOSYNERGISTS OF XANTHOTOXIN¹

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Abstract—The methylenedioxyphenyl-containing (MDP) inhibitors of mixed-function oxidase detoxification enzymes, myristicin, safrole, fagaramide, and isosafrole, occur with xanthotoxin or other toxic furanocoumarins in plants of the families Umbelliferae and Rutaceae. All four MDP compounds have a synergistic effect on the toxicity of xanthotoxin to *Heliothis zea*. Myristicin also increased the phototoxicity of xanthotoxin in the presence of UV light. The term phytosynergist is used to describe plant compounds that are present at concentrations producing no toxic effect by themselves but have a synergistic effect on cooccurring toxins.

Key Words—Xanthotoxin, *Heliothis zea*, Lepidoptera, Noctuidae, myristicin, fagaramide, safrole, isosafrole, synergism, phytosynergist, phototoxicity.

INTRODUCTION

The occurrence of insecticide synergists in plants has led to speculation that their natural function may be synergism of cooccurring toxins (LaForge and Barthel, 1944; Krieger et al., 1971). This view is supported by experiments in which the insecticide synergist myristicin (Figure 1), at levels present in plants, increased the mortality of first-instar *Heliothis zea* caterpillars fed xanthotoxin (Figure 1), a toxic furanocoumarin occurring with myristicin in many plants of

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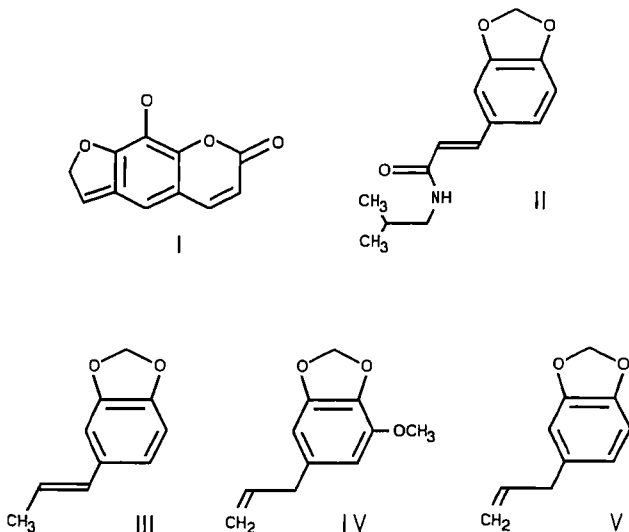


FIG. 1. Xanthotoxin and plant compounds with a synergistic effect on its toxicity: xanthotoxin (I), fagaramide (II), isosafrole (III), myristicin (IV), and safrole (V).

the family Umbelliferae (Berenbaum and Neal, 1985). In this paper, compounds such as myristicin that are present at concentrations producing little or no toxic effect by themselves but which increase the toxicity of cooccurring toxins will be called phytosynergists.

Myristicin, like many other compounds containing a methylenedioxyphenyl (MDP) substituent, is an inhibitor of mixed-function oxidases (MFOs) (Hodgson and Philpot, 1974). MFOs are detoxification enzymes that metabolize many xenobiotics (Hodgson, 1985) and are primarily responsible for the metabolism of xanthotoxin in at least two species of insects (Bull et al., 1984, 1986). The synergistic effect of myristicin on the toxicity of xanthotoxin to *Heliothis zea* is attributed to its inhibition of xanthotoxin metabolism, which increases the effective xanthotoxin concentration in the insect (Berenbaum and Neal, 1985). Xanthotoxin has two types of toxic action on insects, a "dark" toxicity (toxicity in the absence of UV-A light) and phototoxicity (increased toxicity in the presence of UV-A light) (Berenbaum, 1978), which act through different mechanisms (Murray et al., 1982). Both mechanisms are likely to be important under natural conditions; however, only the synergistic effect of myristicin on the dark toxicity of xanthotoxin has been measured (Berenbaum and Neal, 1985). If myristicin works by inhibiting the detoxification of xanthotoxin, however, its activity should be retained under a variety of environmental conditions and be independent of the mode of action of the toxin. Thus, myristicin should

also be a phytosynergist of xanthotoxin phototoxicity in the presence of UV-A light.

Myristicin is not the only MDP-containing insecticide synergist that occurs with xanthotoxin in plant species. Phytochemical records show that safrole and apiole also occur with xanthotoxin in the Umbelliferae (Ceska et al., 1987; Hodisan et al., 1980; Murray et al., 1982; Hegnauer, 1973; Harborne, 1971), and fagaramide, asarinin, and sesamin occur with xanthotoxin in plants of the family Rutaceae (Thoms, 1911; Thoms and Thümen, 1911; Hegnauer, 1973; Murray et al., 1982). Some rutaceous plants also contain safrole and isosafrole along with other toxic furanocoumarins (Chang, 1976; Hegnauer, 1973; Murray et al., 1982). Thus, myristicin is probably not the only plant compound to function naturally as a phytosynergist of xanthotoxin, and there are many toxins other than xanthotoxin that may occur with phytosynergists.

Three insecticide synergists that are MFO inhibitors, fagaramide, safrole, and isosafrole (Figure 1), were tested as synergists of xanthotoxin toxicity to *Heliothis zea*. In addition, the ability of phytosynergists to maintain activity independent of the mode of action of the toxin was studied by measuring the synergistic effect of myristicin on xanthotoxin in the presence as well as in the absence of UV-A light.

METHODS AND MATERIALS

Chemicals. Myristicin was purchased from Saber Labs (Morton Grove, Illinois); fagaramide, safrole, and isosafrole from Aldrich Chemical Co. (Milwaukee, Wisconsin); and xanthotoxin from Sigma Chemical Co. (St. Louis, Missouri).

Bioassays. Neonates of the corn earworm, *Heliothis zea* (Lepidoptera; Noctuidae), from a culture maintained by Dr. G. Waldbauer at the University of Illinois, were used for testing the oral toxicities of all allelochemicals and combinations of allelochemicals. *H. zea* was chosen for study because it is a generalist feeder, it is susceptible to the toxic effects of xanthotoxin, and because myristicin has a synergistic effect on xanthotoxin toxicity to this insect (Berenbaum and Neal, 1985). Chemicals were administered by incorporation into an artificial diet (Berenbaum and Neal, 1985) in which wheat germ is the only undefined ingredient. In order to ensure uniformity, xanthotoxin (dissolved in acetone) was mixed with the alphacel component of the diet and dried prior to its incorporation into the diet. Myristicin, fagaramide, safrole, and isosafrole were added to hot diet in acetone solution (1% of the total wet weight) prior to gelling.

Neonates (30 per treatment) were placed individually on 200 mg diet in a 1.5-ml polypropylene test tube (Biorad, Richmond, California) and reared in a

25°C incubator under cool, white, fluorescent lights (16 hr light–8 hr dark). When screened by the polypropylene tube, these lights delivered less than 0.2 mW/m² of either UV-A (290–400 nm) or UV-B (200–290 nm) light as measured by a UVX radiometer with UVX 36 and UVX 31 sensors (UVP Inc., San Gabriel, California). Caterpillars were monitored until they died or molted to second instar. Toxicity of the MDP compounds was first measured at 1000 ppm (weight/wet weight diet); concentrations at and above 1000 ppm have been recorded for some of the MDP compounds in plants (Lichtenstein and Casida, 1963; Shulgin, 1966). For compounds toxic at 1000 ppm, lower concentrations were tested until a no-effect level was found. To test for synergism, the toxicity of xanthotoxin alone was compared to its toxicity in the presence of MDP compounds. LC₅₀ values were determined from log dose/probit mortality plots (SAS Institute Inc., 1982). LC₅₀ values were considered significantly different if the 95% confidence intervals did not overlap. Synergistic ratios (SR) were calculated as the LC₅₀ of the toxin divided by the LC₅₀ of the toxin with synergist.

For measuring the effects of myristicin–xanthotoxin combinations on *H. zea* in the presence and absence of UV-A light at simulated natural levels, larvae were reared in 50-mm × 5-mm internal diameter Pyrex test tubes stoppered with a cotton applicator stick. Pyrex transmits solar UV wavelengths (290–400 nm) but eliminates nonsolar UV wavelengths. Natural UV-A light intensities were simulated using two Sylvania BLB bulbs that produced between 1700 and 1950 mW/m² UV-A light and between 0.4 and 0.7 mW/m² UV-B at the surface of the tubes as measured with a UVX radiometer; a 16 hr light–8 hr dark photoperiod was used. For the no-UV-light treatments, filters opaque to wavelengths less than 400 nm were used to exclude UV light. Toxicity was evaluated as described above.

RESULTS

In the absence of xanthotoxin, neither myristicin nor fagaramide showed any toxicity at the highest dose tested. Both safrole and isosafrole were toxic at 1000 ppm, each killing 33% of the first-instar caterpillars. Safrole killed 10% at 700 ppm and was nontoxic at 400 ppm. Isosafrole killed 4% at 400 ppm and was nontoxic at 100 ppm. Therefore, in tests for synergistic effects on xanthotoxin, myristicin and fagaramide were used at 1000 ppm, safrole at 400 ppm, and isosafrole at 100 ppm.

Xanthotoxin has an LC₅₀ of 21,000 ppm (Table 1). The LC₅₀ values for combinations of xanthotoxin with myristicin, fagaramide, safrole, and isosafrole are 3200 ppm, 8300 ppm, 5900 ppm, and 8500 ppm, respectively. These values are all significantly different from the LC₅₀ of unsynergized xanthotoxin. Synergistic ratios are 6.5, 2.5, 3.5, and 2.5 for myristicin, fagaramide, safrole,

TABLE 1. SYNERGISM OF TOXICITY OF XANTHOTOXIN TO FIRST-INSTAR *Heliothis zea* BY MYRISTICIN, FAGARAMIDE, SAFROLE, AND ISOSAFROLE

Xanthotoxin (ppm in diet)	Mortality (%) ^a				
	Control	1000 ppm myristicin	1000 ppm fagaramide	400 ppm safrole	100 ppm isosafole
0	0	0	0	0	0
1000	NT	23	10	10	20
2000	3	43	13	37	13
5000	13	67	17	40	37
10000	20	97	67	77	57
15000	30	NT	NT	NT	NT
20000	47	NT	NT	NT	NT
LC ₅₀	21,000	3,200	8,300	5,900	8,500
95% CL ^b	17,000-29,000	2,200-4,200	6,800-11,000	4,600-7,800	6,500-13,000
SR ^c		6.5	2.5	3.5	2.5

^aN ≥ 30. NT = not tested.

^b95% CL = 95% confidence limit; LC₅₀ values are significantly different ($P < 0.05$) if the 95% confidence intervals do not overlap.

^cSR = synergistic ratio.

and isosafole, respectively (Table 1) but are not directly comparable because of differences in the dosages.

In the presence of UV light, myristicin significantly increases the LC₅₀ of xanthotoxin 3.6-fold (Table 2). UV light also has a significant effect on the toxicity of xanthotoxin-myristicin combinations, increasing the LC₅₀ 2.7-fold (Table 2).

DISCUSSION

Fagaramide, safrole, and myristicin all fit the definition of a phytosynergist; they have a synergistic effect on a cooccurring toxin at levels that are nontoxic. Isosafole, which is also a synergist of xanthotoxin, may be a phytosynergist for other cooccurring toxins. These compounds do not necessarily act exclusively as phytosynergists in all plants that produce them. For example, levels of myristicin in some plant parts (Shulgin, 1966) have an insecticidal effect in some insects (Lichtenstein and Casida, 1963). However, insecticidal concentrations of these compounds are rare in plants compared to sublethal concentrations. The occurrence of these compounds at sublethal concentrations suggests a primary role as a phytosynergist.

TABLE 2. SYNERGISM OF XANTHOTOXIN TOXICITY TO FIRST-INSTAR *Heliothis zea* BY COMBINATIONS OF MYRISTICIN (1000 ppm) AND UV-A LIGHT

Xanthotoxin (ppm in diet)	Mortality (%) ^a		
	No myristicin, UV-A light	Myristicin, no UV-A light	Myristicin with UV-A light
100	NT	NT	30
250	17	10	47
500	40	20	57
1000	43	47	63
2500	50	70	70
LC ₅₀	2,200	1,600	600
95% CL	1,300-15,000	1,300-2,100	0-1,300
		Synergistic ratios	
	No myristicin/myristicin (UV-A present)	3.6	
	No UV-A/UV-A (myristicin present)	2.7	

^a $N \geq 30$. NT = not tested.

^b95% CL = 95% confidence limit. LC₅₀ values are significantly different ($P < 0.05$) if the 95% confidence intervals do not overlap.

Berenbaum and Neal (1985) proposed that myristicin acts as an inhibitor of xanthotoxin detoxification and thereby increases the effective concentration of xanthotoxin in the insect. As predicted, myristicin is a synergist of xanthotoxin in both the presence and absence of near UV light. Phytosynergists may further enhance the effectiveness of phototoxins against nocturnal feeders by extending the length of time a phototoxin remains in an insect. This could prevent elimination of a phototoxin prior to the next photophase.

Little is known about the extent to which phytosynergists occur in the plant kingdom. While MDP compounds are widespread, most have not been tested as inhibitors of MFOs. The ecological advantages to phytosynergists are as yet undefined. Possible benefits include a reduction of the energy expenditure for defense or establishing a more stable defense (Berenbaum and Neal, 1986). Such properties would be desirable in economically important crops where phytosynergists may have practical benefits.

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CHEMICAL COMPOSITION AND FUNCTION OF
METAPLEURAL GLAND SECRETION OF THE ANT,
Crematogaster deformis SMITH (HYMENOPTERA:
MYRMICINAE)¹

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Abstract—The secretion of the hypertrophied metapleural gland of the ant *Crematogaster deformis* contains a mixture of phenols, consisting mainly of 3-propylphenol, 3-pentylphenol, 3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein), 5-propylresorcinol, and 5-pentylresorcinol. The secretion is released, as a repellent, when the highly vulnerable petiolar-postpetiolar region of the abdomen is attacked by enemy ants. In addition, small amounts of the secretion are released regularly to serve as an antiseptic, which is considered the original function of the gland. The secretion also has some insecticidal properties.

Key Words—*Crematogaster deformis*, Hymenoptera, Myrmicinae, ant, defensive allomone, repellent, metapleural gland, 3-propylphenol, 3-pentylphenol, 3,4-dihydro-8-hydroxy-3-methylisocoumarin, mellein, 5-propylresorcinol, 5-pentylresorcinol.

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INTRODUCTION

The exocrine glands of ants are known to produce a remarkable variety of compounds, and extensive investigations have been carried out on their glandular chemistry (Attygalle and Morgan, 1984). However, the metapleural glands located in the thorax, although found in most ants, have received little attention. In the few species that have been studied, only carboxylic acids have been identified. Some of these acids show antibiotic properties (Maschwitz et al., 1970; Schildknecht and Koob, 1970, 1971).

Crematogaster deformis possesses a relatively enlarged metapleural gland, similar to other species of *Crematogaster* belonging to the subgenus *Physocrema*. It had been indicated that the secretions of this gland are used to repel enemies (Maschwitz, 1974). Here, we describe in detail the chemical composition and function of the glandular secretion. For the first time, a unique mixture of phenols, hitherto not described from any arthropod source, has been found.

METHODS AND MATERIALS

Sources of Insect Materials. A large colony of *C. deformis* was dwelling in an inaccessible hole in a tree near the Gombak Valley Field Study Centre of the University of Malaya. A few artificial feeding sites made of honey-water mixtures were provided nearby. Polymorphic workers, which were day-active, were attracted in great numbers within a short time to these sites. The ants were caught, kept in moistened plastic containers, and observed from time to time for 10 days. When the ants were loosely held by the thorax region, with a pair of forceps, they released their metapleural gland secretion. These droplets were withdrawn by glass capillaries (Morgan and Tyler, 1977) and sealed in larger capillaries for later examinations.

A colony of *C. scutellaris* was collected in southern France. Its secretions were isolated and sealed in the same way as described for *C. deformis*.

Gas Chromatography (GC). Gas chromatography was carried out on a Hewlett-Packard 5890 instrument fitted with a 25-m \times 0.22-mm fused-silica capillary column coated with SE-54. The oven was held at 60°C for 2 min and programmed at 6°C/min to 260°C. Further analysis was performed on a United Technologies-Packard 438A instrument equipped with a 25-m \times 0.22-mm fused-silica column coated with SP-2340. The oven was kept at 60°C for 4 min and increased at 4°C/min to 195°C. The samples dissolved in hexane were chromatographed by splitless injection.

Micropreparative gas chromatography was performed on a Hewlett-Packard 5750 instrument fitted with a 2-m \times 4-mm glass column packed with 4%

OV-17 on 80–100 mesh Gas Chrom Q. The effluent was split 99:1 (trap–FID) by an all-glass splitter (Baker et al., 1976). The glandular secretion, dissolved in CS₂, was injected and the effluent volumes corresponding to each major component were trapped in metal U-tubes cooled in Dry Ice. The material trapped was washed directly with CDCl₃ (400 μl) into NMR sample tubes (5 mm) and 400 MHz FT-NMR spectra were measured by a JEOL JNM GX-400 instrument.

Gas Chromatography–Mass Spectrometry (GC-MS). The identification of the major components was achieved by GC-MS. A Finnigan 9502 gas chromatograph, fitted with a Grob-type split-splitless injector, linked to a Finnigan 3200E quadrupole mass spectrometer with a Data System 6000 was used. A fused-silica capillary column (SE-54, 25 m × 0.22 mm) was directly coupled to the mass spectrometer. The carrier gas was helium at 1 ml/min. The oven was held at room temperature for 4 min and programmed at 6°C/min to 260°C.

Acetylation of Extract. The metapleural gland extract in hexane (5 μl) was mixed with an ethereal solution (5 μl) containing 5% acetic anhydride and 5% pyridine. The mixture was left overnight and examined by GC and GC-MS.

Chemicals. 5-Propylresorcinol was kindly provided by Prof. Ayer (University of Alberta). Small samples of mellein were sent by Prof. Mori (University of Tokyo) and Dr. Brophy (University of New South Wales). 5-Pentylresorcinol (olivitol) was purchased from Aldrich Chemicals.

3-Propylphenol (peak 1) was synthesized by a Wittig reaction of ethylenetriphenylphosphorane with 3-methoxybenzaldehyde. The 3-(1-propenyl)-anisole thus obtained [yield 79%, bp 120–130°C/15 torr, kugelrohr (128–129°C/20 torr, Hudson and Robinson, 1941)] was hydrogenated to 3-propylanisole [yield 68%, bp 110–120°C/20 torr, kugelrohr (92°C/11 torr, Parkes, 1948)] using 5% Pd/C as catalyst, and the latter was hydrolyzed with acetic acid–HBr to give 3-propylphenol, peak 1 [yield 15%, bp 80–90°C/20 torr, kugelrohr (100–102°C/6–7 torr, Hartung and Crossley, 1934), mass and [¹H]NMR spectra in Table 1].

3-Pentylphenol (peak 2) was synthesized by a similar procedure, using butylenetriphenylphosphorane for carbonyl olefination. The 3-(1-pentenyl)-anisole formed [yield 84%, bp 73–76°C/0.01 torr (92–99°C/1 torr, Alles et al., 1942)] was hydrogenated to 3-pentylanisole [yield 92%, bp 70–72°C/0.05 torr (97–98°C/3 torr, Alles et al., 1942)] and subsequently hydrolyzed to 3-pentylphenol (peak 2) [yield 82%, bp 69–72°C/0.05 torr (99–100°C/1 torr, Alles et al., 1942), mass and [¹H]NMR spectra in Table 1].

Behavioral Studies. In order to study the attacking behavior of *C. deformis*, a worker of *Oecophylla smaragdina* was placed in an assembly of *C. deformis* at a honey–water feeding site. Similarly, the defence behavior was studied by placing a *C. deformis* ant at an assembly of *O. smaragdina*. The latter species was selected as the test ant species because it occupies the same habitat

TABLE 1. CHEMICAL COMPOSITION OF METAPLEURAL GLAND SECRETION OF *C. deformis*: SUMMARY OF ANALYTICAL EVIDENCE FOR STRUCTURE ASSIGNMENT

Peak ^a	Identification	Mass spectral data <i>m/z</i> (%)			NMR ^b [δ]
		Natural product	Acetylated product		
1	3-Propylphenol	136(M ⁺ , 30), 135(8), 108(50), 107(100), 106(29), 94(6), 91(8), 77(39)	178(M ⁺ , 7), 137(7), 136(74), 121(17), 108(68), 107(100), 106(24), 94(7), 91(13), 77(35), 43(50)	0.93 (3H, t, <i>J</i> = 7.3 Hz), 2.53 (2H, t, <i>J</i> = 7.4 Hz), 4.90 (1H, s), 6.63 (1H, d, <i>J</i> = 8.0 Hz), 6.64 (1H, s), 6.75 (1H, d, <i>J</i> = 7.6 Hz), 7.14 (1H, t, 7.6 Hz)	
		164(M ⁺ , 17), 121(13), 108(100), 107(68), 106(16), 91(9), 77(29)	206(M ⁺ , 3), 164(24), 122(9), 121(12), 108(100), 107(59), 91(10), 77(31), 43(45)		
		178(M ⁺ , 100), 160(43), 149(17), 135(17), 134(88), 133(12), 132(19), 121(5), 106(22), 105(15), 104(22), 78(20), 77(16), 51(12)	220(M ⁺ , 0), 178(86), 160(49), 149(13), 134(100), 133(27), 106(20), 105(27), 104(25), 78(33), 77(48), 51(43), 43(92)	1.51 (3H, d, <i>J</i> = 6.4 Hz), 2.91 (2H, d, <i>J</i> = 7.0 Hz), 4.72 (1H, 6 lines), 6.66 (1H, d, <i>J</i> = 0.9 Hz), 6.68 (1H, d, <i>J</i> = 0.9 Hz), 7.39 (1H, t), 11.01 (1H, s)	
2	3-Pentylphenol	164(M ⁺ , 17), 121(13), 108(100), 107(68), 106(16), 91(9), 77(29)	206(M ⁺ , 3), 164(24), 122(9), 121(12), 108(100), 107(59), 91(10), 77(31), 43(45)		
		178(M ⁺ , 100), 160(43), 149(17), 135(17), 134(88), 133(12), 132(19), 121(5), 106(22), 105(15), 104(22), 78(20), 77(16), 51(12)	220(M ⁺ , 0), 178(86), 160(49), 149(13), 134(100), 133(27), 106(20), 105(27), 104(25), 78(33), 77(48), 51(43), 43(92)		
3	Mellein	135(17), 134(88), 133(12), 132(19), 121(5), 106(22), 105(15), 104(22), 78(20), 77(16), 51(12)	220(M ⁺ , 0), 178(86), 160(49), 149(13), 134(100), 133(27), 106(20), 105(27), 104(25), 78(33), 77(48), 51(43), 43(92)		
		105(15), 104(22), 78(20), 77(16), 51(12)	78(33), 77(48), 51(43), 43(92)		
4	5-Propylresorcinol	152(M ⁺ , 49), 137(14), 124(100), 123(51), 67(18), 69(20)	194(M ⁺ , 0), 152(97), 137(12), 123(37), 124(80), 51(22), 43(100)		
		123(51), 67(18), 69(20)	123(37), 124(80), 51(22), 43(100)		
5	5-Pentylresorcinol	180(M ⁺ , 22), 138(11), 137(11), 125(6), 124(100), 123(28), 69(9), 67(7)	264(M ⁺ , 4), 222(17), 180(95), 138(36), 137(17), 124(100), 123(68), 43(91)	0.87 (3H, t, <i>J</i> = 7.0 Hz), 2.47 (2H, t, <i>J</i> = 7.8 Hz), 5.20 (1H, broad s), 6.17 (1H, t, <i>J</i> = 2.3 Hz), 6.25 (2H, d, <i>J</i> = 2.2 Hz)	
		125(6), 124(100), 123(28), 69(9), 67(7)	138(36), 137(17), 124(100), 123(68), 43(91)		
		69(9), 67(7)	123(68), 43(91)		

^aThe peak numbers refer to Figure 1. All the compounds identified showed identical retention times to those given by authentic samples, on SE-54 and SP-2340 columns.

^b400 MHz FT-NMR spectra were recorded using about 10 to 20-μg samples. Signals not given were masked by impurity peaks.

^cMaterial available was insufficient for a satisfactory NMR spectrum.

and is considered an important competitor of *C. deformis*. The repellent effect of the metapleural gland secretion was studied by offering isolated thoraces of *C. deformis*, or chopped grasshopper parts smeared with one gland-equivalent of the secretion, to an assembly of *O. smaragdina* at a feeding site. The behavior of the ants was observed for 20 min after placing each lure. Similarly, grasshopper parts smeared with 1 μ l of 3-pentylphenol, or honey-water containing 1, 0.1, 0.01, or 0% 3-pentylphenol, were offered to colonies of *O. smaragdina* and *Pheidole* sp. Likewise, 2 gland-equivalents of the secretion were applied to individual mealworm larvae (*Tenebrio molitor*), which were offered to an European pied flycatcher (*Ficedula hypoclea*).

In order to study the toxic effects of the secretion, either 1 gland-equivalent of the metapleural gland secretion or 1 μ l of 3-pentylphenol was applied, by a glass capillary, onto the gaster or head of *O. smaragdina* workers. Thirty ants were subjected to each treatment and observed for 20 hr.

The release of alarm behavior was investigated by presenting crushed body parts, such as a head, excised mandibular glands, thorax, or gaster, on a piece of filter paper, near a feeding site of *C. deformis*. The ants were observed for 1 min after each stimulus was presented. The experiment was repeated six times for each stimulus.

RESULTS

Release of Glandular Secretions. The workers of *C. deformis*, which were kept in closed containers, released small quantities of their metapleural gland secretion even when they were not being disturbed. The secretion is released from the orifices of the reservoirs, which have no closing mechanism. The presence of the secretion could be recognized by its characteristic tarlike smell, when the lid of the container was opened.

When an ant was loosely held, with a pair of forceps, by its antenna, leg, or head, sometimes the release of minute quantities of the metapleural secretion could be observed. However, when the ant was gripped directly by the thorax, or gaster, a large colorless or somewhat violet droplet appeared at the gland orifice. Due to the lipophilic nature of the secretion, it soon spread over the cuticle around the orifice. When the ants were treated more roughly, seized by the head or legs, they released their very sticky brownish-red-colored Dufour gland contents and tried to smear the secretion on the pair of forceps by which they were held.

Chemical Analysis of Secretion. The metapleural gland secretion withdrawn into glass capillaries had a strong phenolic smell. The analysis of the extract by gas chromatography revealed the presence of five major components (Figure 1). The highly polar nature of these components was evident from the

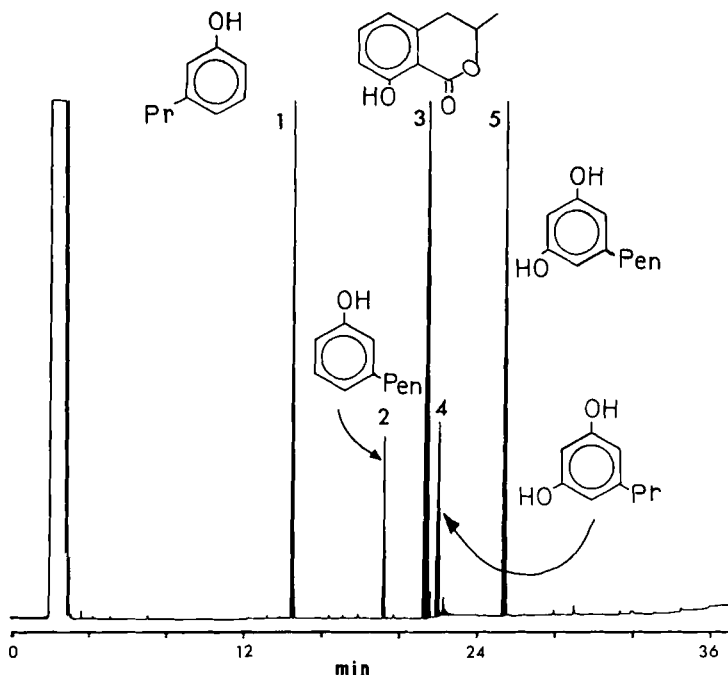


FIG. 1. Volatiles in the metapleural gland secretion of *C. deformis*. Chromatogram obtained on a 25-m \times 0.22-mm fused-silica capillary column coated with SE-54. Oven temperature was held at 60°C for 2 min and programmed at 6°C/min to 260°C. The sample in hexane was introduced by splitless injection. Peak numbers refer to those in Table 1.

large shifts in retention times observed when a chromatogram obtained on a nonpolar column (SE-54) was compared to one on a polar column (SP-2340). All five components showed much longer retention times on the polar column. Complete mass spectra were obtained by GC-MS for all components, and satisfactory NMR spectra were recorded for the three major compounds. The results are summarized in Table 1.

In order to verify the presence of phenols, the mixture was acetylated. Components 1, 2, 4, and 5 underwent complete acetylation, and new peaks corresponding to the products appeared in the chromatogram. The peak 3 was only partially acetylated under the conditions used. The GC-MS analysis of the acetylated mixture showed that components 1, 2, and 3 were monoacetylated, while 4 and 5 were diacetylated. The degree of change in the retention times also indicated this.

The mass spectrum of peak 2 was identical to that known for 3-pentyl-

phenol (Gorfinkel et al., 1969). Both 2- and 4-pentylphenols have the base peak at m/z 107; only the 3-pentylphenol shows the base peak at m/z 108. Although the spectrum of peak 1 was very similar to those from 2-propylphenol and 4-propylphenol (Stenhagen et al., 1974), there were significant differences. For example, the spectrum of peak 1 has a significant peak at m/z 121 (13%) which is absent in those of 2- and 4-propylphenols. Also, the peak at m/z 108 is more prominent in the natural product (50%) than those of 2- and 4-isomers (less than 8%). It appeared that such a prominent even-mass ion at m/z 108 can arise only from a 3-isomer. The pentyl and higher derivatives studied by Gorfinkel et al. (1969) show a similar behavior. In fact, if the alkyl group is pentyl or larger, the m/z 108 peak becomes the base peak. The NMR spectrum (Table 1) could establish that the peak 1 is a *n*-propyl compound and not an isopropyl derivative. 3-Propylphenol was synthesized, and it was chromatographically and spectroscopically identical to the natural product.

Peak 3 could be identified immediately by its mass spectrum, which was congruent with that known for 3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein) (Brand et al., 1973). The mass and NMR spectra (Table 1) and the retention time of an authentic sample were identical to those from the natural compound.

To be consistent with mass spectroscopic and acetylation results, peak 5 must be a pentylresorcinol. According to the deductions of Occolowitz (1964), only the 1,3,5-isomer of such a compound can yield an even-mass ion at m/z 124. The properties of an authentic sample of 5-pentylresorcinol matched well with those of the natural product. By analogy, peak 4 was suspected to be 5-propylresorcinol, which was later proved correct by comparison with an authentic sample.

The absolute amount of the phenols present per ant varied widely from 2 to 20 μg per sample. Although the relative amounts of compounds also showed a broad variation, the chromatogram shown in Figure 1 can be considered typical.

The mixture of phenols found seems characteristic to the species *C. deformis*. For example, such phenols were not detected in the metapleural glands of *C. scutellaris* or *C. borneensis* (Attygalle, Fiala, and Maschwitz, unpublished observations).

Defensive and Attacking Behavior. The attacking behavior of *C. deformis* could be studied by introducing an alien ant, such as *O. smaragdina* or *Diacamma rugosa*, into a large assembly of *C. deformis* workers at a feeding site. The workers attacked the alien instantly and bit into its body appendages. Spread-eagled by this attack, the alien was unable to move, and in this posture it was dragged along the recruitment trail. The use of metapleural or Dufour gland secretions was not observed during these attacks. On the other hand, when a worker of *C. deformis* was placed in an assembly of *O. smaragdina*, it

too was attacked immediately, either by biting into the body appendages or the body itself. When two or more workers of *O. smaragdina* got hold of the appendages, they pulled and spread-eagled the *C. deformis* worker, immobilizing it in such a way that it was unable to use its defensive glands. Subsequently, it was killed slowly by other ants that reinforced the attack. However, if the attackers initially bit into the head or neck region, *C. deformis* could bend its highly mobile gaster tip forward and smear the attacker with sticky Dufour gland secretion. In 10 of 15 such attacks observed, the contaminated *O. smaragdina* worker stopped the attack and began to clean itself. On the contrary, if the attackers first bit into the posterior thorax, the coxae, the petiolar region, or the proximal gaster region, *C. deformis* released, in defense, its metapleural gland secretions. This immediately stopped the attack. In 18 of 20 cases observed, the contaminated *O. smaragdina* workers immediately started an intensive and prolonged cleaning process. They wiped their heads on the ground and cleaned their mouth-parts with their legs.

The strong repellent effect of the metapleural gland secretion was also evident when we placed isolated thoraces of *C. deformis*, or chopped grasshopper parts, smeared with the secretion, at a feeding site of *O. smaragdina*. While the uncontaminated parts were carried away instantly at the first contact, no objects smelling of the metapleural secretion were taken. Similar results were obtained with grasshopper parts contaminated with 3-pentylphenol. Likewise, honey-water containing 1 or 0.1% 3-pentylphenol was refused by *Pheidole* sp., while untreated solutions were consumed by hundreds of ants. However, a 0.01% solution was also accepted. *O. smaragdina* refused even the uncontaminated solutions. Moreover, the secretion did not manifest any repellent effect when contaminated mealworm larvae were offered to a European pied flycatcher. The bird consumed six mealworms, one after the other, without any hesitation.

Besides the repellent effects, the secretion also shows some toxicity to insects. Table 2 shows the results obtained by treating workers of *O. smaragdina* with the secretion of *C. deformis* or 3-pentylphenol.

TABLE 2. MORTALITY CAUSED BY METAPLEURAL GLAND SECRETION AND 3-PENTYLPHENOL^a

Part of <i>O. smaragdina</i> subjected to treatment	Number of dead ants counted after treating with		
	1 Metapleural gland equivalent of <i>C. deformis</i> .	1 μ l of 3-pentylphenol	Control (water)
Head	16	8	0
Gaster	6	30	0

^a30 Workers of *O. smaragdina* were subjected to each treatment and observed for 20 hr.

TABLE 3. ALARM BEHAVIOR RELEASED IN ITS WORKERS BY CRUSHED BODY PARTS OF *C. deformis*^a

	Crushed body part presented				
	Head	2 Excised mandibular glands	Thorax	Gaster	Control ^b
Total number of ants showing alarm behavior ^c	252	230	24	27	23

^aThe ants were observed for 1 min, after presenting each stimulus on a piece of filter paper to an assembly of *C. deformis* workers near a feeding site.

^bA piece of filter paper.

^cThe total number, after repeating each presentation six times.

Although the metapleural secretion has repellent and some toxic effects towards insects, it has no alarm pheromone properties. Alarm behavior (i.e., fast movements, attraction, opening of the mandibles, and biting behavior) was exclusively released by the mandibular gland secretion (Table 3).

DISCUSSION

The metapleural glands are found in most ants. As far as is known from the few species that have been analyzed, the glandular secretion contains certain carboxylic acids that show antibiotic and antiseptic properties. These acids are supposed to act as topical antiseptics that suppress the growth of fungi and bacteria on the body surface of the adults and brood, and on the nest material (Maschwitz et al., 1970; Maschwitz, 1974; Schildknecht and Koob, 1971; Hölldobler and Engel-Siegel, 1984).

C. deformis regularly releases small amounts of its phenolic metapleural gland secretion to maintain its body and nest hygiene. The bactericidal effect of phenols is well known. The presence of an alkyl chain tends to increase lipid solubility, and from the investigations conducted by Ayer et al. (1983), it is clear that the bactericidal activity increases, up to a certain point, as the length of the side chain increases. Phenols, very similar to those found in the gland are often used in proprietary antiseptics.

The phenolic compounds, except mellein, identified in *C. deformis*, have been reported from biological sources only on rare occasions. 3-Propylphenol is an attractant for tsetse flies and has been isolated in the urine of the host buffalo (Hassanali et al., 1986). It is also found in tobacco smoke (Arrendale, 1984). 3-Propylresorcinol has been found in a lichen (Chamy et al., 1985), and it has been claimed to be present in oakmoss extracts (Gavin and Tabacchi,

1975); however, the published mass spectrum does not correspond to ours obtained from an authentic sample. Long-chain alkyl phenols and resorcinols are characteristic of the oils of Anacardiaceae (Bestmann et al., 1987; Skopp et al., 1987; Oocolowitz, 1964). The only similar compound known from ants is 5-methylresorcinol (orcinol) (Blum et al., 1982a)

Mellein is a fairly ubiquitous compound with antibiotic properties. It was first found as a fungal metabolite (Ayer and Shewchuk, 1986, and references therein), and later its presence was shown in ants (Brand et al., 1973; Brophy et al., 1981; Bellas and Hölldobler, 1985), termites (Blum et al., 1982b), and moths (Baker et al., 1981; Nishida et al., 1982; Kunesch et al., 1987).

Producing an antibiotic secretion may be the sole primary function of the metapleural gland secretion in ants having a "normal-sized" gland; however, at least in those species possessing a hypertrophied (exceptionally enlarged) gland, additional functions can be expected. *C. deformis* has such an enlarged gland, and this hypertrophic development is accompanied by chemical specialization. None of the phenols found in this unique mixture, except mellein, has been previously isolated from any arthropod source. From our observations it is clear that *C. deformis* uses this secretion also as a defensive allomone. In fact, this secondary function is the more important role of the secretion. The strong-smelling secretion has a powerful repellent, and even toxic, effect against enemy ants. The slender petiolar-postpetiolar region of *C. deformis* is highly vulnerable to enemy attacks of biting. The metapleural secretion exclusively provides the chemical defense of this region. The head-neck region, body appendages, and gaster tip of the workers are protected by the defensive secretions from the hypertrophied Dufour gland. This secretion can be applied, at the aforementioned body regions, employing the gaster, which is highly mobile. A highly mobile gaster is a characteristic feature of the genus *Crematogaster*. Thus, *C. deformis* is protected by a two-gland chemical defense system, which has, however, not taken over the alarm function. The alarm pheromone is produced in the mandibular gland. In contrast, in *C. (Physocrema) inflata*, it is produced in the metapleural gland (Maschwitz, 1974). The injecting part of the sting of *C. deformis* is atrophied, similar to those in other species of *Crematogaster*.

It appears that a change has occurred, during phyletic development, from a more primitive system of injecting the hydrophilic venom from the poison gland, to a more advanced defense system using surface-active lipophilic secretions of the Dufour gland. The lipophilic and highly sticky Dufour gland secretion can entangle the enemy. The metapleural secretion is repellent, or sometimes even lethal, to the enemy. Such "new" chemical weapons employing surface-active allomonnes are common in higher Formicidae (Buschinger and Maschwitz, 1986). Although venom gland secretions of ants are often potent toxicants, the injection of the venom into the body of the enemy ant is very

difficult, if not impossible, especially when the opponent is highly mobile, small, or mass-attacking. For fighting against such an enemy, an instantly applicable repellent or a sticky fluid, such as those utilized by *C. deformis*, is much more effective.

Furthermore, the petiolar region of myrmicine and some other ants are usually protected against biting ants by small but sharp epinotal spines. These spines, found in most other *Crematogaster* species, are lacking in *C. deformis* because the petiolar region is well-protected by the metapleural gland secretion. A similar development can be seen in *C. inflata*. It also lacks epinotal spines and produces a sticky glue-like defensive secretion in its enormously enlarged metapleural gland (Maschwitz, 1974).

The genus *Crematogaster* demonstrates an astonishing variety in its defensive chemistry and biology and will be the subject of further investigations.

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SUNFLOWER VOLATILES INVOLVED IN HONEYBEE DISCRIMINATION AMONG GENOTYPES AND FLOWERING STAGES

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Abstract—In order to define the part of olfactory cues in the selective behavior of honeybees, observation on their foraging behavior was carried out on various sunflower genotypes in parallel with chemical analysis of aromatic extracts of the genotypes. Foragers show a preference for the early stages of flowering and, when they are given a choice between couples of parental lines of two commercial hybrids, Marianne and Mirasol, they are randomly distributed on Mirasol parents, but they prefer the female line of Marianne. The comparison of relative proportions of compounds among aromagrams obtained from head space trapping from the two couples of genotypes, reveals (1) a phenological stage effect for 17 compounds among 144 indexed compounds for Marianne lines and for 18 among 136 indexed compounds of Mirasol lines; most of these compounds exhibit higher relative proportions in the early flowering stages, which is related to plant attractiveness towards honeybees; (2) a sex effect for 33 compounds among 144 for Marianne lines and for 14 compounds among 136 for Mirasol lines; further semiquantitative analyses reveal a sex effect for only eight compounds of 134 for Marianne lines and 20 compounds of 250 for Mirasol lines, which represents less than 10% of the indexed compounds. These discriminatory compounds were partly identified by coupled GC-MS. Possible relations between such phenological and genotypical volatile fluctuations and forager attraction are discussed.

Key Words—Sunflower volatiles, genotype effect, phenology effect, honeybee, Hymenoptera, Apidae, foraging behavior, olfactory discrimination, hybrid seed production.

INTRODUCTION

The sunflower crop (*Helianthus annuus* L.) has spread widely in recent years and is now the second largest oilseed crop in the world (Putt, 1978). This increase is linked to improvement in varieties through hybrid production using cytoplasmic sterility (Leclercq, 1969). Hybrid production is then dependent on pollen carriage from male to female lines and the transfer is mainly carried out by insects (Putt, 1940; Free and Simpson, 1964), and more particularly by honeybees (*Apis mellifica* L.) (Radford and Rhodes, 1978; Parker, 1981). However, field observations have shown that low seed yields were linked to a selective foraging behavior of honeybees (Shein *et al.*, 1978; Freund and Furgala, 1982), and it has been established that olfactory parameters are determinant for forager attraction to a food source (Frisch, 1967; Masson, 1983).

Little work dealing with the chemistry of sunflower volatiles has been reported in the literature. Most authors have studied the chemistry of higher molecular weight constituents, such as sesquiterpenic lactones (Iriuchijima *et al.*, 1966; Morimoto, 1966), diterpenic acids (Pyrek, 1970; Stipanovic *et al.*, 1979), fatty alcohols (Gracian and Arevelo, 1980), germacranolides (Ortega *et al.*, 1970; Ohno and Mabry, 1979), sterols (Homberg and Schiller, 1973), and triterpenes (Kasprzyk and Janiszowska, 1971). Gershenzon *et al.* (1981) studied the chemistry of terpenoid constituents of *Helianthus*, and Eckert *et al.* (1973) identified the sesquiterpenic hydrocarbon β -gurjunene and several terpenoids in unrefined sunflower oil.

The first data dealing with more volatile compounds are reported by Popescu (1979, 1982). Popescu *et al.* (1979) identified 19 monoterpenes, and Karawya *et al.* (1980) pointed out five terpenic hydrocarbons and five oxygenated terpenoids; this work was completed by Flath *et al.* (1985), who reported 14 monoterpene hydrocarbons, 25 oxygenated monoterpenes, and several sesquiterpenic hydrocarbons. These authors carried out steam distillation on different parts of cut sunflower heads. Etievant *et al.* (1984) set up direct solvent extraction and head space collection of volatiles from cut heads of various sunflower genotypes. Analysis of extracts by combined gas chromatography and mass spectrometry led to 84 constituents being reported, among which 20 terpenic hydrocarbons, 9 alcohols, 3 phenols, 6 esters, and 19 oxygenated compounds were identified. Moreover this work emphasized qualitative differences in the composition of polar fractions of the genotypes.

In the present work, we have tried to define the part of olfactory cues in selective behavior of the honeybee by the parallel observation of their foraging behavior towards different sunflower genotypes and chemical analysis of aromatic extracts of the corresponding genotypes. In order to carry out the chemical analysis of extracts as close as possible to volatile emissions perceived by insects around the sunflower disk, extracts were obtained after head space trap-

ping of volatiles emitted by living heads. By comparing extracts from different plant genotypes and/or phenological stages, we have tried to point out volatiles likely to be used by honeybees as cues for behavioral discrimination.

METHODS AND MATERIALS

Plant Material. Experiments were carried out during two successive years on flower disks (male and female lines) of two sunflower hybrids (Marianne and Mirasol). These hybrids have highly different seed yields (low yields for Marianne, which is no longer cultivated in France, and high yields for Mirasol, used as the standard for varieties for which selection is in progress).

During the first year, aroma collection and behavioral observations were carried out on the two parental lines and at three phenological stages, I, II, and III, corresponding to 1/3, 2/3, and 3/3, respectively, of disk florets in blossom. During the second year, aroma samples were collected on each genotype at stage II.

Behavioral Observations. Foraging behavior of insects was observed in forced pollination conditions, previously described (Pham-Delegue *et al.*, 1986). A hive containing about 10,000 worker bees gathered around a 1-year-old queen and provided with brood and food combs was placed under a tunnel facing a couple of sunflower parental lines. Forager distribution was recorded on 40 heads per genotype with three observations per day, every other day alternately for each variety throughout the blossom period (six days for Marianne lines and five days for Mirasol lines). The number of foragers per head, as well as the stage of blossom, were taken into account. The frequentation level of a line was estimated by the mean number of foragers per 10 sunflower heads. Attractiveness of parental lines was noted for each couple of lines, by Student's *t* test.

Chemical Analysis. Plant volatile emissions were collected and concentrated using a head space trapping method adapted to sunflower heads.

Purification of Reagents. Solvents were distilled prior to use, and their purity was checked by gas chromatography, as described by Etievant and Bayonove (1983). Porapak-Q (120–150 mesh) was purified for 6 hr using a Soxhlet extractor with nanograde hexane. After solvent evaporation at ambient temperature, further drying was carried out by heating to 110°C for 2 hr. After packing (2.2 g Porapak-Q in 10-cm-long × 1-cm ID glass tubing). Porapak-Q traps were further conditioned at 230°C for 12 hr in a flow of nitrogen (1–2 liters/hr).

Trapping of Volatiles. Volatile compounds emitted by one sunflower head were collected on a conditioned Porapak-Q trap placed between the sampling bag (polyvinyl fluoride film, Tedlar, Dupont de Nemours int., S.A.) and a blade pump (Figure 1). An air intake connected to a coal trap for air purification was

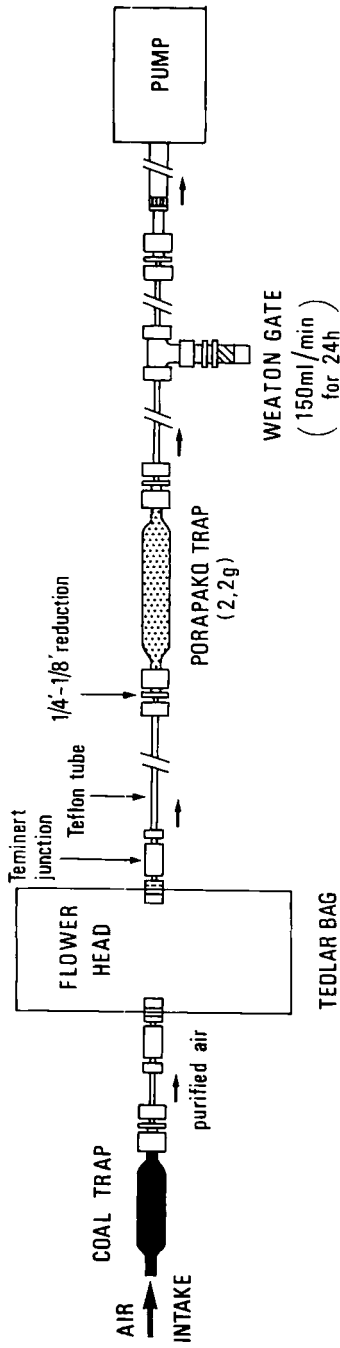


FIG. 1. Scheme of head space trapping of volatiles from living sunflower heads.

set up in the bag. All tubes and connections were made of Teflon. Air flow was adjusted to 150 ml/min by using a Weaton needle valve set between the trap and the pump. Trapping was performed for 24 hr.

Desorption of Volatiles from Traps. Humidity condensed in the traps was first eliminated by a flow of dry nitrogen (150 ml/min for about 1 hr at ambient temperature) applied in the opposite direction to the one chosen for collection of volatiles. Volatiles were then desorbed by percolation of 2 × 25 ml Freon 11. The solution was then concentrated to a volume of about 500 μ l through a Dufton column. Extracts were kept in sealed tubes in a freezer (-25°C) for further analysis.

Separation and Identification of Constituents. Volatile constituents of the aromatic extract were separated according to conditions described by Etievant *et al.* (1984); analyses were carried out with a Girdel 300 gas chromatograph fitted with a split-splitless injector, a flame ionization detector, and a SE-52 glass WCOT capillary column (37 m × 0.4 mm ID; film thickness 0.6 μ m). The gas carrier was helium (17.1 ml/min), temperatures of the injector and detector were, respectively, 230 $^{\circ}\text{C}$ and 220 $^{\circ}\text{C}$, and the temperature of the oven was programmed from 40 to 180 $^{\circ}\text{C}$ at 3 $^{\circ}\text{C}/\text{min}$.

When necessary, identifications were carried out by coupling gas chromatography and mass spectrometry according to conditions previously described by Etievant and Bayonove (1983). Samples were analyzed by both electron impact and chemical ionization using NH_3 as the reagent gas. Acquisition and processing of mass spectra were set by a connected computer (Digital Equipment Corporation PD P8) and identification of compounds was made automatically, referring to a spectra library (National Institutes of Health, Environmental Protection Agency), and checked manually by comparing with published spectra.

Statistical Analysis. The quantitative study of volatile differences according to the genotype (male versus female) and to the flowering stage (Stages I, II, and III) was first carried out on samples collected the first year. Extracts from each stage and each genotype were twice injected in the gas chromatograph, without any internal standard added. The integrated area of each peak was referred to the total area of all indexed peaks of the chromatogram; resulting data obtained from chromatographic profiles of each variety were then submitted to an univariable analysis of variance with two controlled factors (sex, stage), according to a cross-model with interaction. The analysis was carried out with a MINI6 microcomputer (CII) at the INRA of Dijon, using the ANVARM program from the statistical library AMANCE (Bachacou *et al.*, 1981).

The analysis of the stage effect was followed by a multiple comparison of means, using the Scheffe method. Thus the amounts of each constituent that differed significantly according to the stage were classified.

In a second step, analysis was focused on the genotype effect from samples collected at stage II of blossom, with three repetitions for male lines, two repetitions for female lines, and two injections for each sample; 2-hexanone (1 mg), added before concentration of extracts, was used as a standard. The integrated area of each indexed peak was referred to the standard peak area. In order to compare male to female aromagrams, univariable analysis of variance was set up, using the UNFAL program, which calculated for each constituent the mean proportion and the standard deviation as well as the F value of the Fisher test.

RESULTS

The behavioral data are reported in Figure 2, and a foraging preference for the flowering stages I and II appears for all genotypes. Moreover, the foragers exhibit a different foraging behavior among the two couples of genotypes: they are randomly distributed on the parents of the Mirasol hybrid, while they selectively visit the parents of the Marianne hybrid, with a great preference for the female. This emphasizes the honeybees' ability to discriminate between plant genotypes.

During the first step of the physicochemical analysis of lines sex and stage

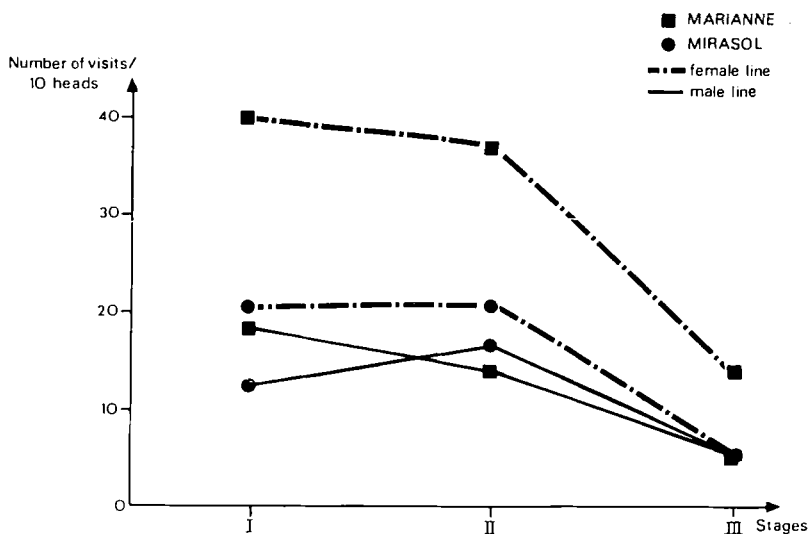


FIG. 2. Foraging activity of honeybees according to seasonal phenology and to genotypes (stages I-III: 1/3 to all disk florets in blossom).

effects for each couple of plant lines, 144 components were indexed for Marianne parents and 136 components for Mirasol lines. Among these indexed components, 56 of 144 exhibit significant differences with a 0.05 probability level according to sex for Marianne lines (with a higher amount in the female line for 38 components), and 41 components for Mirasol lines (with a higher amount in the female line for 28 components). However, some of those components show a significant sex/stage interaction, which makes interpretation difficult because genotype emissions may fluctuate according to flowering stages. These components showing a sex/stage interaction were rejected, i.e., 23 for Marianne lines and 27 for Mirasol lines. A high proportion of remaining discriminatory components exhibits a higher value for the male line (23 among 33 for Marianne lines, and all 14 for Mirasol lines) (Figure 3).

Concerning the stage effect, it appears that 38 of 144 for Marianne and 54 of 136 for Mirasol present significant differences with a 0.05 probability level. Among these, 17 for Marianne and 18 for Mirasol show a stage effect without any interaction with the sex effect (Figure 4). Most of them exhibit a higher relative proportion at stage I for Marianne lines (9 of 17) and at stage II for Mirasol lines (13 of 18). Such data are related to plant attractiveness towards honeybees, according to flowering stages.

In order to develop the study of possible relationships between honeybee attraction and the relative amounts of volatiles, new experiments were set up; stage III was excluded because of the low number of honeybee visits (see Figure 2). Between the other stages, stage II was preferred to stage I, in order to get a better flowering concordance among male and female lines for simultaneous volatile collection.

Physicochemical analysis was carried out using an internal standard to obtain semiquantitative data, which allow easier interpretation of peak variations. A reference index was reported independent for each variety (134 for Marianne lines and 250 for Mirasol lines were indexed). Statistical analysis was applied to these indexed components, comparing male with female lines. Significant differences appear for eight components of 134 for Marianne lines and for 20 components of 250 for Mirasol lines (Figures 5 and 6), despite some high values of variation coefficients ($> 50\%$ for values obtained for peaks 71 and 105 in the male line of Marianne, and for values obtained for peaks 10, 43, and 179 in the male line and peak 211 in the female line of Mirasol). These variations in the amounts of some components may be related to a low reproducibility of the emission between different flower heads and to a lower precision of the integration of small peaks.

However, it has to be stressed that intergenotype differences are not based on the quantitatively dominant components; qualitative differences (according to detection limits of the GC technique) arise for three components of eight for

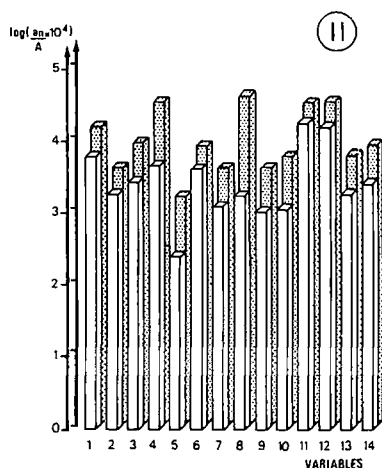
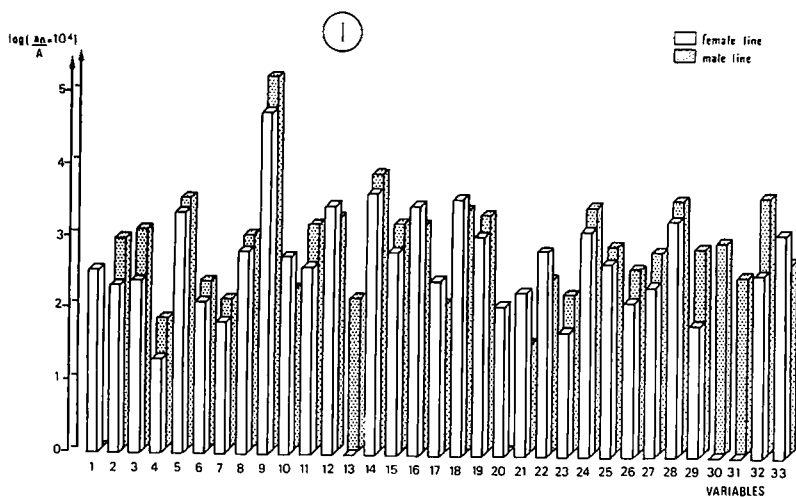


FIG. 3. Intergenotype differences in volatiles among Marianne (I) parental lines and Mirasol (II) parental lines. Variables: indexed components with significant differences according to sex, without interaction with stage effect (orthogonal variance analysis with two controlled factors: sex, stage).

$$\log \frac{(a_n \times 10^4)}{A}$$

expresses the ratio of the peak area (a) of a given component (n) to the total area (A) of all the indexed components of the chromatographic profile. *y axis value of the component 13 in Marianne lines is = -3.95.

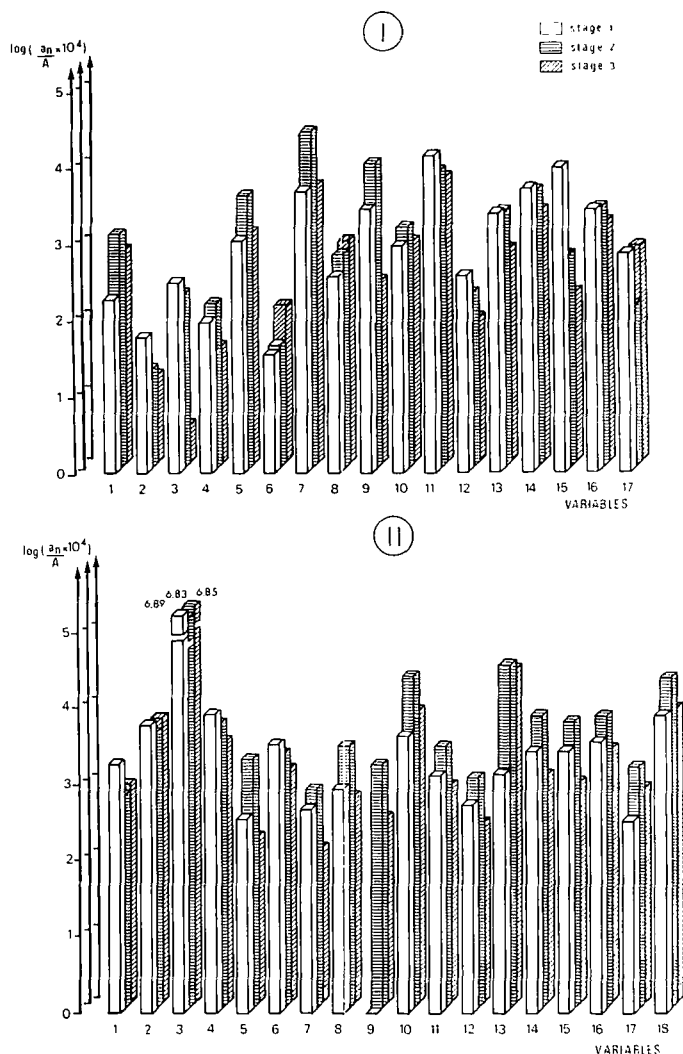


FIG. 4. Phenological differences in volatiles for Marianne lines (I) and Mirasol lines (II).

Marianne and for 12 components of 20 for Mirasol, the volatile component lacking in the female line in most of the cases. Moreover, the amounts of most volatiles are higher in the male line of the Mirasol variety (for 16 components of the discriminatory ones).

As reported in Figures 5 and 6, identification of some of the dis-

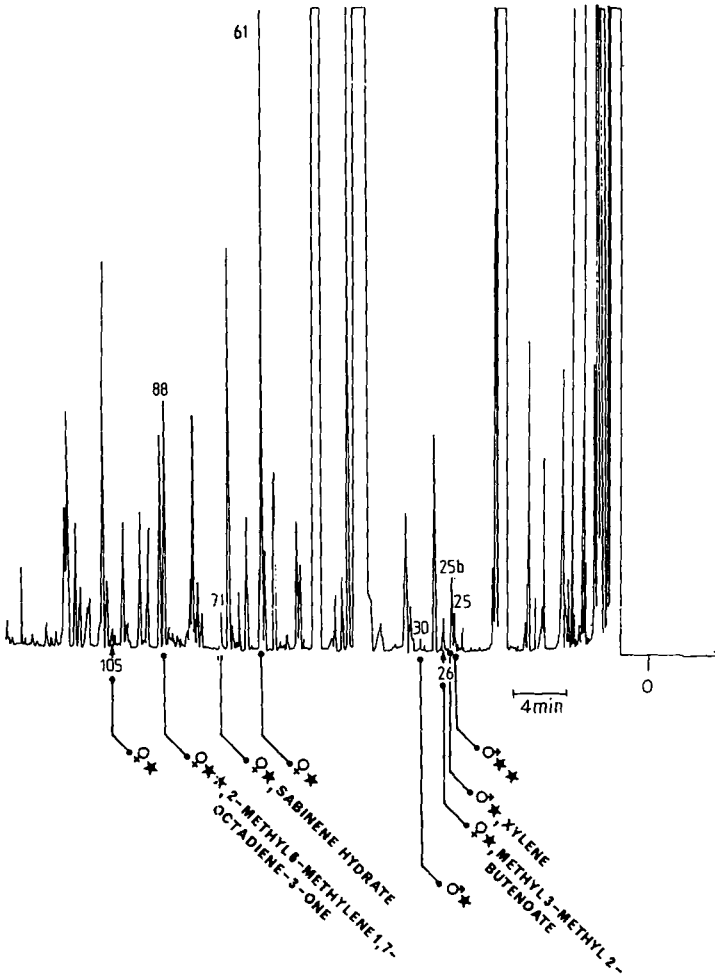


FIG. 5. Discriminatory components among male and female parental lines of Marianne from semiquantitative analysis. ♂, ♀, indicate the line where the component has the highest amount. Intergenotype differences are obtained from an univariable analysis of variance with one rank factor (1,5 ddl). * $P < 0.05$; ** $P < 0.01$.

criminatory components was then elucidated by GC-MS coupling; xylene, methyl 3-methyl-2-butenate, limonene, sabinene hydrate, and 2-methyl-6-methylene-1,7-octadien-3-one have been identified among the eight intergenotype discriminatory components of Marianne parents; methylcyclopentane, 2,3,4-trimethylhexane, decahydro-1,6-dimethylnaphthalene,

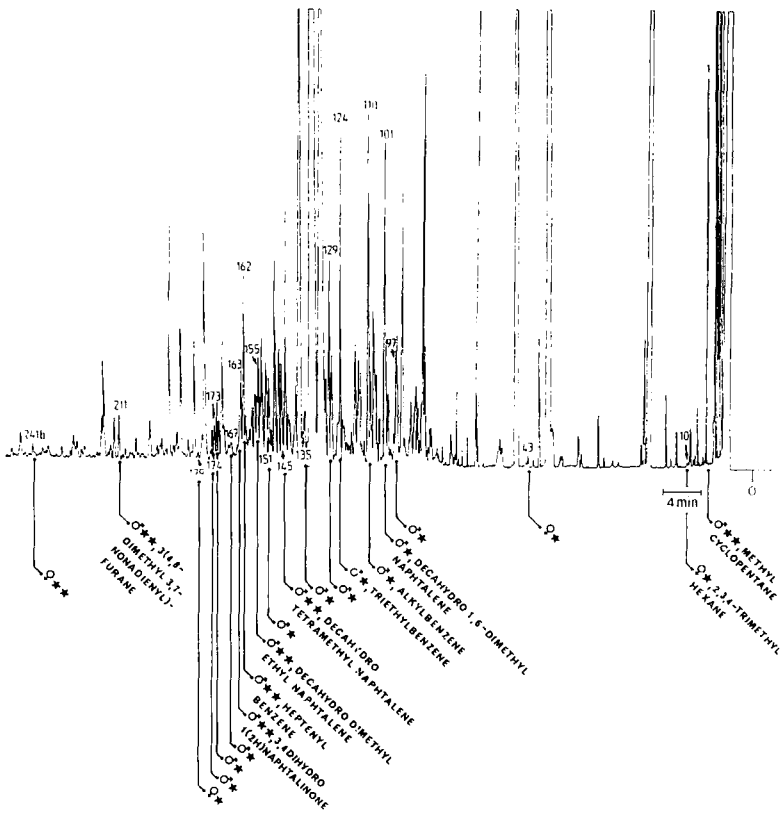


FIG. 6. Discriminatory components among male and female lines of Mirasol (see legend to Figure 5).

alkylbenzene, triethylbenzene, decahydrotetramethylnaphthalene, decahydro-dimethylethyl-naphthalene, 1-heptenylbenzene, 1,4-dihydro-1-(2H)naphthalenone, and 3(4,8-dimethyl-3,7-nonadienyl)-furan were part of the 20 discriminatory components of Mirasol parental lines.

DISCUSSION

Honeybees are opportunistic foragers whose tendencies for innate responses can be largely obliterated by learning, and mainly by olfactory conditioning. Manning (1957) observed that a change of scent induced a stronger discrimination than a change of flower pattern or shape, and several authors (e.g. Frisch, 1967; Koltermann, 1969) concluded that scent was more important in condi-

tioning honeybees than color, shape, or time of day. Although we are aware that continued foraging by honeybees is dependent on the reward of nectar or pollen, plant selection is based on aroma differences.

The data of honeybee visitation on flowers at different blossom stages indicated a decrease at the later stage (stage III) for all the genotypes considered. These results agree with those of Benedek and Manninger (1972), Delaude *et al.* (1979), and Drane *et al.* (1982), who mentioned a higher attractiveness at the beginning and in the middle of sunflower flowering. It has been shown in the present work that the late flowering stage corresponded to the lowest amounts of stage discriminatory components. Thus a seasonal phenology of volatile chemicals appears in sunflower genotypes, which affects their attractiveness to honeybees. This fact underlines the possible use by honeybees of some volatile components representative of plant flowering. Variations may inform foragers of growth stages and of the corresponding food availability, since visitation by bees is closely linked to the largest caloric reward (Tepedino and Parker, 1982), which decreases throughout the flowering (Simidichev, 1976; Fonta *et al.*, 1985).

Although seasonal variations in the composition of the volatile oil had already been quantified in several plant aromas [e.g., variations in terpenes production of *Tanacetum vulgare* L. volatile oil (Rudloff and Underhill, 1965)], little work had been done to establish relations between volatile fluctuations and insect attraction. Some evidence for such abilities of the honeybee to select plant stage according to volatiles had been pointed out in the soybean (Robacker *et al.*, 1982, 1983), which suggested that some aroma chemicals may communicate flower-readiness information to pollinators.

As for the varietal effect, our data, particularly from semiquantitative analysis, points out differences among coupled genotypes restricted to less than 10% of the chromatographic profiles. This fact emphasizes honeybees' discrimination abilities, since they are able to make long-range selection among genotypes in Marianne lines, using these slight differences. It must also be noted that discriminatory components seem to be chemically different for the two couples considered. Moreover, these differences are not systematically used for selection, since honeybees exhibit preferences only between Marianne parents, which is closely related to differences in glucidic food reward (Fonta *et al.*, 1985). Thus, in a situation of choice, discriminatory components only became significant cues for selective orientation after association with the best reward during the conditioning process.

It had been shown previously that honeybees were able to recognize sunflower aroma from a limited chemical pattern whose active constituents were representative of the whole aroma (Pham-Delegue *et al.*, 1986). Referring to this work, it appears that the chemicals already identified from this active fraction (Etievant *et al.*, 1984; Pham-Delegue *et al.*, 1986) are different from the

discriminatory intergenotype volatiles identified in the present work. Thus, the hypothesis suggested is that foragers may recognize a flower aroma from a limited typical fraction of the blend, while they may use very slight differences, out of this common chemical pattern, to discriminate between genotypes and choose the best food source. Such differences may not be used when the two genotypes are equally supplied, as for Mirasol lines, where both genotypes have equivalent glucidic composition (Fonta *et al.*, 1985). Currently the effort of identification of active compounds is carried out with a synchronous combination of chemical and biological recordings (Thiery *et al.*, in preparation). This method should make it possible to define a few molecular criteria responsible for sunflower attractiveness to honeybees.

Moreover, the genetic basis for such compounds, common to a plant genus or specific to a given genotype has to be checked. Thus, at it has been shown in *Mentha genus* (Hefendehl and Murray, 1976), that the genetic aspect of volatile biosynthesis may be elucidated in order to create the proper combination of genes necessary to accumulate large amounts of single important components. In this research area, Gershenzon *et al.* (1981), reviewing the terpenoid chemistry of sunflowers, have already suggested that some terpenoids, such as sesquiterpenic lactones and diterpenes, are possible sources of resistance to some of the most serious pests.

The present type of research, which needs the cooperation of chemists, physiologists, plant breeders, and geneticists, should lead to the definition of chemicals involved in plant attractiveness to pollinators and likely to be included in plant selection programs for crop improvement through controlled cross-pollination.

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PLANT PHOTSENSITIZERS: A Survey of Their Occurrence in Arid and Semiarid Plants from North America

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Abstract—Various plants native to arid and semiarid habitats throughout the southwestern United States, Baja California, and northern Mexico were bioassayed for phototoxic natural products. Approximately 115 species representing 57 genera and eight plant families were assayed for phototoxic activity by standard antimicrobial techniques using *Escherichia coli* and *Saccharomyces cerevisiae*. Phototoxic constituents were extracted from numerous members in the Asteraceae (Compositae) and occurred with highest frequency among species of the subtribe Pectidinae (tribe Heliantheae). Extracts of *Pectis*, the largest genus in the Pectidinae, had substantial light-activated biocidal action despite the paucity of acetylenic thiophenes, the phototoxins characteristic of most other genera in the subtribe. Leaf resin from the creosote bush [*Larrea tridentata* (Sesse & Mol. ex DC.) Coville; Zygophyllaceae], a dominant desert shrub, possessed potent antimicrobial activity in the absence of light; however, the toxicity of this extract was slightly enhanced in the presence of UVA irradiation. Phototoxic antimicrobials were not detected in extracts of selected species from the Asclepiadaceae, Chenopodiaceae, Hydrophyllaceae, Lamiaceae, Polygonaceae, or Solanaceae.

Key Words—Photosensitizers, arid/semiarid plants, Asteraceae, Zygophyllaceae, Asclepiadaceae, Chenopodiaceae, Hydrophyllaceae, Lamiaceae, Polygonaceae, Solanaceae.

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INTRODUCTION

Plant "photosensitizers" or "phototoxins" are chemicals produced by members of at least 30 plant families that adversely affect a wide variety of organisms in the presence of sunlight (Downum, 1986). A partial listing of susceptible organisms includes among others: viruses, phytopathogenic bacteria and fungi, nematodes, and herbivorous insects (see reviews by Towers, 1984, 1986; Downum, 1986; Downum and Rodriguez, 1986; Downum and Nemeč, 1987). In addition to these organisms, Campbell et al., (1982) demonstrated that the growth of various non-phototoxin-containing plants could be inhibited by treatment with photosensitizers common to many species of the Asteraceae (Compositae). Such broad-spectrum biological activity suggests that plant phototoxins may be involved with plant defense against a wide range of potentially deleterious or competing organisms in nature.

Although phototoxic phytochemicals have been isolated from a variety of plants, little is known about their occurrence (or biological activity) in species that evolved under high-light conditions where phototoxic action seemingly would provide a most effective plant defense. The present report is an initial effort to establish the prevalence of photosensitizers in plants that grow in environments exposed to high levels of solar irradiation (i.e., the Chihuahuan, Mojave, and Sonoran deserts of North America). Standard antimicrobial techniques were used for these preliminary survey studies, but the broad-spectrum toxicity associated with many of the previously studied plant phototoxins suggests that they may be responsible for a variety of defensive plant responses, in addition to their *in vitro* antimicrobial effects.

METHODS AND MATERIALS

Plant Material. Bulk collections for extraction and bioassay as well as voucher specimens were collected from various regions of Mexico and the southwestern United States as indicated in Table 1. Plants were air-dried in the field as much as possible and then transported to the laboratory for thorough drying (50°C for five days). Dried, powdered matter (5 g) from each species was extracted with MeOH (50 ml) for 7–10 days at room temperature under darkened conditions. The resulting extracts were filtered and concentrated to approximately 1 ml by rotary evaporation. Concentrated crude extracts were stored at –20°C until they could be bioassayed.

Species of *Pectis* were handled differently to minimize the loss of potential phototoxins, which was reported previously (Downum et al., 1985). These plants were transported from the field on ice and homogenized in MeOH within

TABLE 1. PLANTS FROM ARID AND SEMIARID REGIONS OF UNITED STATES AND MEXICO BIOASSAYED FOR PHOTOTOXIC AND ANTIBIOTIC ACTIVITY WITH *E. coli* AND *S. cerevisiae*^a

Plants	Collection site	<i>E. coli</i>	<i>S. cerevisiae</i>
Asclepiadaceae			
<i>Asclepias</i>			
<i>A. subulata</i> Decne.	Baja Calif.	—	—
Asteraceae			
Anthemideae			
Artemisia ^b			
<i>A. dracunculus</i> L.	Baja Calif.	—	—
<i>A. californica</i> Less.	Baja Calif.	—	—
<i>A. tridentata</i> Nutt.	Nevada	—	—
<i>A. ludoviciana</i> Nutt. subsp. <i>incompta</i> (Woot.) Keck	Calif.	—	—
<i>A. bigelovii</i> A. Gray in Torr.	Nevada	—	—
<i>Chrysanthemum</i> ^b			
<i>C. coronarium</i> L.	Baja Calif.	—	—
Astereae			
<i>Chrysothamnus</i> ^b			
<i>C. nauseosus</i> (Pall.) Britt. subsp. <i>consimilis</i> (Greene) Hall & Clements	Nevada	—	—
<i>C. paniculatus</i> (A. Gray) Hall.	Nevada	—	—
<i>C. viscidiflorus</i> (Hook.) Nutt subsp. <i>puberulus</i>	Nevada	—	—
<i>Ericameria</i> ^c			
<i>E. linearifolia</i> (DC.) Urbatsch & Wussow	Baja Calif.	—	—
<i>Hazardia</i> ^c			
<i>H. brickellioides</i> (S.F. Blake) Clark	Nevada	—	—
<i>H. linearifolius</i> DC.	Baja Calif.	—	—
<i>H. squarrosus</i> (H. & A.) Greene var. <i>grindelioides</i> (DC.) Clark	Calif.	—	—
<i>Isocoma</i> ^c			
<i>I. acradenia</i> (Greene) Greene	Nevada	—	—
<i>I. veneta</i> (H.B.K.) Greene	Calif.	—	—
<i>Machaeranthera</i> ^c			
<i>M. pinnatifida</i> (Hook.) Shinnors subsp. <i>goodingii</i> (A. Nels.) Turner & Hartman	Nevada	—	—
<i>M. tortifolia</i> (A. Gray) Cronq & Keck	Arizona	—	—
Lactuceae			
<i>Malacothrix</i> sp. ^c	Calif.	—	—
Eupatorieae			
<i>Hofmeisteria</i> ^c			
<i>H. fasciculata</i> (Benth.) Walp. var. <i>fasciculata</i>	Baja Calif.	—	—
<i>H. crassifolia</i> S. Wats.	Sonora	—	—

TABLE 1. Continued

Plants	Collection site	<i>E. coli</i>	<i>S. cerevisiae</i>
Heliantheae			
Ambrosiinae			
<i>Ambrosia</i> ^{b,d}			
<i>A. ambrosioides</i> (Cav.) Payne	Arizona	+	+
<i>A. camphorata</i> (Greene) Payne	Sonora	+	+, anti
<i>A. chenopodifolia</i> (Benth.) Payne	Baja Calif.	+	anti
<i>A. confertiflora</i> DC.	Sonora	-	-
<i>A. cordifolia</i> Payne	Arizona	+, anti	+
<i>A. deltoides</i> (Torr.) Payne	Arizona	anti	+
<i>A. dumosa</i> (A. Gray) Payne	Sonora	+	+, anti
<i>A. eriocentrata</i> (A. Gray) Payne	Nevada	-	+
<i>A. trifida</i> L.	Arizona	-	-
<i>Dicoria</i> ^c			
<i>D. canescens</i> A. Gray	Baja Calif.	anti	-
<i>Hymenoclea</i> ^c			
<i>H. salsola</i> Torr. & Gray	Baja Calif.	-	-
<i>Parthenice</i> ^c			
<i>P. mollis</i> A. Gray var. <i>penninsularis</i> Sauck	Arizona	-	-
Baeriinae			
<i>Eriophyllum</i> ^b			
<i>E. lanatum</i> (Pursh) Forbes	Calif.	-	-
<i>Lasthenia</i> sp. ^{b,d}			
<i>L. coronaria</i> (Nutt.) Ornduff	Baja Calif.	-	-
Chaenactidinae			
<i>Bahia</i> ^c			
<i>B. absinthifolia</i> Benth.	Arizona	-	-
<i>Chaenactis</i> ^{b,d}			
<i>C. glabriuscula</i> DC.	Calif.	-	-
<i>Hulsea</i> ^c			
<i>H. californica</i> Torr. & Gray	Baja Calif.	-	-
<i>Palafoxia</i> ^{b,d}			
<i>P. linearis</i> var. <i>glandulosa</i> B.L. Turner & M.I. Morris	Baja Calif.	-	-
Coreopsidinae			
<i>Coreocarpus</i> ^c			
<i>C. dissectus</i> (Benth.) S.F. Blake	Baja Calif.	-	-
<i>C. parthenioides</i> Benth. var. <i>parthenioides</i>	Baja Calif.	-	-
<i>C. paniculatus</i> (A. Gray) Hall	Calif.	-	-
<i>C. viscidiflorus</i> (Hook.) Nutt.	Calif.	-	-
<i>Thelesperma</i> ^b			
<i>T. filifolium</i> (Hook.) A. Gray	Texas	-	+
<i>T. megapotamicum</i> (Spreng.) O. Ktze.	Arizona	-	+
Ecliptinae			
<i>Encelia</i> ^{b,c}			
<i>E. ravenii</i> Wigg.	Baja Calif.	-	+
<i>E. farinosa</i> A. Gray var. <i>farinosa</i>	Baja Calif.	-	+
<i>E. frutescens</i> (A. Gray) A. Gray	Baja Calif.	-	-
<i>E. ventorum</i> Brandege	Baja Calif.	-	anti

TABLE 1. Continued

Plants	Collection site	<i>E. coli</i>	<i>S. cerevisiae</i>
<i>Heliopsis</i> ^b			
<i>H. parviflora</i> A. Gray var. <i>rubra</i> (Fish.) Wigg.	Arizona	-	-
<i>Verbesina</i> ^{b,d}			
<i>V. dissata</i> A. Gray	Baja Calif.	-	-
<i>V. enceloides</i> (Cav.) A. Gray var. <i>exauriculata</i> Robins. & Greenm.	Baja Calif.	-	-
<i>V. palmeri</i> S. Wats.	Baja Calif.	-	-
<i>Zinnia</i> ^b			
<i>Z. grandiflora</i> Nutt.	Arizona	-	-
Gaillardiiinae			
<i>Baileya</i> ^c			
<i>B. multiradiata</i> Harv. & Gray	Calif.	-	-
<i>Psilostrophe</i> ^c			
<i>P. cooperi</i> (A. Gray) Greene	Baja Calif.	-	-
Galinsoginae			
<i>Bebbia</i> ^c			
<i>B. juncea</i> (Benth.) Greene var. <i>juncea</i>	Baja Calif.	-	-
Helianthinae			
<i>Helianthus</i> ^b			
<i>H. gracilentus</i> A. Gray	Baja Calif.	-	+
<i>H. niveus</i> (Benth.) Brandey. subsp. <i>niveus</i>	Baja Calif.	-	-
<i>Heliomeris</i> ^c			
<i>H. multiflora</i> Nutt. var. <i>nevadensis</i> (A. Nels.) Yates	Nevada	-	-
<i>Viguiera</i> ^d			
<i>V. deltoidea</i>			
var. <i>chenopodina</i> (Greene) S.F. Blake	Baja Calif.	-	+
var. <i>deltoidea</i>	Baja Calif.	-	-
<i>V. dentata</i> (Cav.) Spreng.	Sinaloa	-	-
<i>V. laciniata</i> A. Gray	Baja Calif.	-	-
<i>V. microphylla</i> Vasey & Rose	Baja Calif.	-	-
<i>V. purissimae</i> Brandegee	Baja Calif.	-	-
<i>V. tomentosa</i> A. Gray	Baja Calif.	-	-
Madiinae			
<i>Adenothamnus</i> ^c			
<i>A. validus</i> (Brandegee) Keck	Baja Calif.	-	-
<i>Calycadenia</i> ^b			
<i>C. tenella</i> (Nutt.) Torr. & Gray	Baja Calif.	-	-
<i>Hemizonia</i> ^{b,d}			
<i>H. fasciculata</i> (DC.) Torr. & Gray	Baja Calif.	-	-
Melampodiinae			
<i>Melampodium</i> spp. ^{b,d}	Texas	-	-
Pectidinae			
<i>Adenophyllum</i> ^{d,s}			
<i>A. [Dyssodia] porophylloides</i> A. Gray	Arizona	+	+
<i>Chrysactinia</i> ^d			
<i>C. mexicana</i> A. Gray	Texas	+	+

TABLE 1. Continued

Plants	Collection site	<i>E. coli</i>	<i>S. cerevisiae</i>
<i>Dyssoida</i> ^{d,r}			
<i>D. anthemidifolia</i> Benth.	Baja Calif.	+	+
<i>Hymenatherum</i> ^d			
<i>H. acerosum</i> (DC.) A. Gray	Texas	+	+
<i>H. [Dyssodia] pentachaeta</i> (DC.) B.L. Rob. var. <i>belenidium</i> (DC.) Strother ^e	Arizona	+	+
<i>H. tenuilobum</i> DC.	Comm. Source	+	+
<i>Nicolletia</i> ^d			
<i>N. trifida</i> Rydb.	Baja Calif.	+	+
<i>Pectis</i> ^h			
<i>P. canescens</i> H.B.K.	Guerrero	+	+
<i>P. filipes</i> Harv. & Gray var. <i>subnuda</i> Fern.	Arizona	+	+
<i>P. imberbis</i> A. Gray	Arizona	+	+
<i>P. linifolia</i> L. var. <i>hirtella</i> Blake	Michoacan	+	+
var. <i>linifolia</i>	Oaxaca	+	+
<i>P. longipes</i> A. Gray (2N)	Arizona	+	+
(4N)	Arizona	+	+
<i>P. luckoviae</i> Keil	Michoacan	+	+
<i>P. multiflosculosa</i> (DC.) Sch. Bip.	Jalisco	+	+
<i>P. multiseta</i> Benth.	Baja Calif.	+	+
<i>P. papposa</i> Harv. & Gray var. <i>papposa</i>	Arizona	+	+
<i>P. prostrata</i> Cav.	Arizona	+	+
<i>P. purpurea</i> Bdg. var. <i>sonorae</i> Keil	Sinaloa	+	+
<i>P. schaffneri</i> Sch. Bip. ex A. Gray	Oaxaca	+	+
<i>Porophyllum</i> ^b			
<i>P. gracile</i> Benth.	Baja Calif.	+	+
<i>P. ruderales</i> (Jacq.) Cass. var. <i>macrocephalum</i> (DC.) Cronq.	Arizona	+	+
<i>P. scoparium</i> A. Gray	Texas	+	+
Peritylinae			
<i>Amauria</i> ^c			
<i>A. rotundifolia</i> Benth.	Baja Calif.	-	-
<i>Perityle</i> ^c			
<i>P. emoryi</i> Torr.	Baja Calif.	-	-
<i>P. microglossa</i> Benth.	Baja Calif.	-	-
<i>P. megalcephalum</i> var. <i>intricata</i>	Nevada	+	+
Senecioneae			
<i>Senecio</i> ^f			
<i>S. californicus</i> DC. var. <i>californicus</i>	Baja Calif.	-	-
<i>S. douglasii</i> var. <i>monoensis</i> (Greene) Jepson	Baja Calif.	-	-
<i>S. salignus</i> DC.	Arizona	-	-
<i>S. vulgaris</i> L.	Calif.	-	-
Chenopodiaceae			
<i>Atriplex</i> sp.	Baja Calif.	-	-

TABLE 1. Continued

Plants	Collection site	<i>E. coli</i>	<i>S. cerevisiae</i>
<i>Eurotia</i>			
<i>E. lanata</i> (Pursh) Moq.	Baja Calif.	-	-
Hydrophyllaceae			
<i>Phacelia</i>			
<i>P. distans</i> Benth.	Baja Calif.	-	-
Lamiaceae			
<i>Lepechinia</i>			
<i>L. hastata</i> (A. Gray) Epling	Baja Calif.	-	-
<i>Salvia</i>			
<i>S. columbariae</i> Benth.	Baja Calif.	-	-
Polygonaceae			
<i>Antigonon</i>			
<i>A. leptopus</i> Hook. & Arn.	Sonora	-	-
Solanaceae			
<i>Nicotiana</i>			
<i>N. trigonophylla</i> Dunal	Baja Calif.	-	-
Zygophyllaceae			
<i>Guaiaacum</i>			
<i>G. coulteri</i> A. Gray	Sonora	-	-
Kallstroemia			
<i>K. grandiflora</i> Torr. ex A. Gray	Texas	-	-
<i>K. hirsutissima</i> Vail.	Texas	-	-
<i>Larrea</i>			
<i>L. tridentata</i> (Sesse & Moc. ex DC.) Coville	Baja Calif.	+	+
<i>Viscainoa</i>			
<i>V. geniculata</i> (Kell.) Greene var. <i>geniculata</i>	Baja Calif.	-	-

^aWhole plant extracts were concentrated and assayed as described (see Methods and Materials). Presence (+) or absence (-) of inhibitory zones surrounding bioassay disks refers to activity following UV-A (3 hr) and dark incubation for 24 hr. Growth inhibition resulting from antibiotic action (not photoinduced) of an extract is denoted anti.

^bAcetylenes reported from genus (Bohlmann et al., 1973).

^cChemistry unknown.

^dThiophenes reported from genus (Bohlmann and Zdero, 1976, 1979; Bohlmann et al., 1973, 1980, 1983; Downum and Towers, 1983; Downum et al., 1985).

^eChromenes reported from genus (Proksch and Rodriguez, 1983).

^fGenus reported to lack polyacetylenes and thiophenes (Bohlmann et al., 1973).

^gSee Downum et al. (1985) for explanation of nomenclatural assignment.

^hGenus reported to lack acetylenic di- and terthiophenes (Downum and Towers, 1983; Downum et al., 1985).

24 hr of collection. Extracts were bioassayed immediately as their phototoxicity quickly disappeared following homogenization.

Voucher specimens of all species were deposited with Herbaria either at California Polytechnic State University, the University of California at Riverside, or the University of Texas at El Paso.

Microorganisms. *Escherichia coli* B, a gram-negative bacterium, and *Saccharomyces cerevisiae*, a yeast, were routinely used to bioassay plant extracts for antibiotic and/or phototoxic plant metabolites. Stock cultures of *E. coli* were obtained from the American Type Culture Collection (ATCC 23226); cultures of *S. cerevisiae* were obtained from the laboratory of Dr. G.H.N. Towers (University of British Columbia). Stationary phase cultures (18 hr) grown aerobically at 30°C (*S. cerevisiae*) or 37°C (*E. coli*) were used for all bioassays. *S. cerevisiae* was grown in Sabouraud dextrose broth (Difco) while *E. coli* was inoculated into nutrient broth (Difco).

Bioassay Techniques. Concentrated plant extracts (20–50 μ l) were applied to replicate sterile cellulose assay disks and then placed onto lawns of *E. coli* and *S. cerevisiae* as described previously (Downum et al., 1983). Duplicate plates were prepared for all assays. One plate was kept in the dark to determine the antibiotic action of an extract, and UV-A activation or enhancement of bio-cidal action was determined on a second plate that was irradiated by four horizontal UV-A lamps (Sylvania F40BLB; 18 W/m²). The level of irradiation used for the bioassays was approximately half that routinely encountered in the desert environment (measured at 365 nm with a Spectroline model DM-365N ultraviolet meter; Spectronics Corp., Westbury, New York).

RESULTS AND DISCUSSION

Extracts of many plants belonging to the sunflower family (Asteraceae) inhibited the growth of *E. coli* and *S. cerevisiae* cultures as did the leaf extract from the creosote bush *Larrea tridentata* (Zygophyllaceae) (Table 1) when exposed to UV-A irradiation. Several of these plant extracts also inhibited the growth of the bioassay organisms without light activation; however, this activity was less pronounced than the phototoxic activity. A limited number of extracts from species representing the Asclepidaceae, Chenopodiaceae, Hydrophyllaceae, Lamiaceae, Polygonaceae, and Solanaceae failed to elicit any kind of biological activity toward *E. coli* or *S. cerevisiae*.

Plants belonging to the Asteraceae were given preference in our survey because they represent a quantitatively important component of most arid and/or semiarid habitats in North America and because UV-mediated antibiotic activity has been associated with many plants from this large family (Camm et al., 1975). Extracts from approximately 35% of the species surveyed elicited

phototoxic responses from the bioassay organisms. Several species also contained effective antimicrobial agents that did not require light activation; these included five species of *Ambrosia*, one species of *Dicoria*, and one species of *Encelia*. The greatest number of phototoxin-containing plants belonged to the tribe Heliantheae [as revised by Robinson (1981)]. Nearly 50% of the plant extracts from members of this tribe tested positive for light-activated toxins. The Pectidinae, a subtribe within the Heliantheae, is of particular note because extracts from all of the plants in this taxonomic grouping were phototoxic toward both *E. coli* and *S. cerevisiae*.

Various polyacetylenic or thiophenic metabolites are most likely responsible for the phototoxicity of extracts from many members of the Asteraceae. Such chemicals are widely distributed in the family (Bohlmann et al., 1973) and many are potent photosensitizers (McLachlan et al., 1984, 1986; Marchant and Cooper, 1987). Thiophenes, which are biosynthetically derived from acetylenic precursors by sulfide addition (Bohlmann et al., 1973), are also powerful phototoxins (Downum et al., 1982, 1983). Although these phototoxic metabolites occur sporadically throughout the Asteraceae (Bohlmann et al., 1973), they are particularly characteristic of genera in the subtribe Pectidinae (Bohlmann et al., 1973; Downum and Towers, 1983; Downum et al., 1985). *Pectis*, the largest genus in this subtribe, is, however, phytochemically distinct from the remainder of the genera in this taxonomic grouping in that thiophenes have yet to be detected in the 20 or so species examined thus far (Downum and Towers, 1983; Downum et al., 1985). The identity of the chemical constituents responsible for the phototoxicity of *Pectis* extracts remains in question.

Leaf extracts from the creosote bush, *Larrea tridentata* (Zygophyllaceae), contained constituents that were effective biocidal agents in the absence of UV-A. The antimicrobial activity of this extract was enhanced slightly on exposure to UV-A. The creosote bush was the only plant outside of the Asteraceae to elicit both dark and UV-A-enhanced biological activity against *E. coli* and *S. cerevisiae*. Several other Zygophyllaceae genera were also bioassayed (e.g., *Guaiaacum*, *Kallistroemia*, and *Viscainoa*), but antimicrobial activity similar to that mediated by *Larrea* extracts was not observed.

Isolation, identification, and bioactivity studies of the toxic chemicals from *Pectis* spp. and *L. tridentata* are currently underway.

This preliminary study suggests that phototoxic phytochemicals are fairly common among taxonomically diverse members of the Asteraceae. Light-enhanced biocidal activity was also associated with extracts of *Larrea tridentata*, a dominant shrub throughout the Chihuahuan, Mojave, and Sonoran deserts. The presence of light-activated antimicrobial agents in plants that typically grow under high levels of solar irradiation suggests that such allelochemicals may mediate important interactions between photosensitizer-containing plants and potentially deleterious organisms in these environments. The prev-

absence of phototoxic phytochemicals in other high-light environments (e.g., tropical and subtropical habitats) is currently being investigated.

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GERANIUM DEFENSIVE AGENTS. IV. Chemical and Morphological Bases of Resistance

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Abstract—Insect-resistant and -susceptible geranium plants (*Pelargonium xhortorum*) were chemically and morphologically compared to assess the relative importance of resistance components. Glandular and nonglandular trichome density and quantification of trichome exudate (anacardic acids) from inbred resistant and susceptible lines were determined. Three different extraction procedures were employed to quantify the anacardic acids: (1) capillary solvent extract, (2) leaf wipe, and (3) whole leaf extract. The results support the conclusion that tall glandular trichomes and the exudate they produce are the most important factors in small pest resistance in the geranium. In addition, it was shown that tall glandular trichome densities are less and the ability of the plant to express the anacardic acids as exudate on the trichome head exterior is lacking in the susceptible lines analyzed, even though all lines possessed the capability to synthesize the anacardic acids. A plant line of intermediate resistance character was shown to possess high densities of tall glandular trichomes but resembled susceptible plants in lacking the ability to express the anacardic acids as an exudate in appreciable quantities.

Key Words—Geranium, *Pelargonium xhortorum*, glandular trichomes, anacardic acids, plant defense.

INTRODUCTION

Resistance to mites and insects in the geranium (*Pelargonium xhortorum*) has been shown to be largely chemically based, resulting from a glandular trichome exudate composed of anacardic acids (10'-*o*-pentadecenyl and 12'-*o*-heptadecenyl salicylic acid) (Stark, 1975; Gerhold et al., 1984; Craig et al., 1986).

Structural details of these exudate components have been determined (Gerhold et al., 1984; Walters et al., 1988). Gerhold reported that GC analysis of leaf press collections of mite-susceptible plants did not possess glandular trichome exudate, but his collection technique did not extract material from the leaf tissue, only that found on the trichome surface. Using the leaf disk bioassay described by MacDonald et al. (1971), Winner (1975) developed a resistance index, comprised of mortality and fecundity measurements, to group plants into susceptible and resistant phenotypic classes. Using these techniques, Winner concluded that two dominant complimentary genes were required to produce the resistant phenotype.

Geraniums possess four trichome types, a tall and a short glandular trichome and a tall and short spiny-type trichome, which are easily distinguished under a dissecting microscope (Stark, 1975) (Figure 1). Chang et al. (1972) have previously presented evidence that trichome density might be related to mite resistance in the geranium. However, Chang's study was limited in scope and the researchers failed to separate the tall and short trichomes.

The purpose of the research described here is to further illuminate the important heritable aspects of the resistance mechanism by a comparison of the chemical and morphological characteristics of resistant vs. susceptible plants. Glandular and nonglandular trichome density and chemical quantification of the exudate using three different extraction procedures were performed on inbred lines of resistant and susceptible plants in an attempt to measure the possible importance of these characters in the resistance mechanism.

METHODS AND MATERIALS

Plant Materials. The five lines of geraniums used for this study were propagated as cuttings from greenhouse stock maintained in the Horticulture Department of The Pennsylvania State University. Two of the inbred lines (71-17-7 and 71-17-1) had previously been determined to be mite resistant (Stark, 1975), and two of the lines (71-10-1 and 71-15-4) had been determined to be mite susceptible (Winner, 1975). In addition, a line (71-18-6) classified as resistant by Winner, but which appears to exhibit a degree of susceptibility to mite and aphid attack in the greenhouse, was included. Plants were grown in the greenhouse at approximately 21°C and were watered as conditions dictated. Soluble fertilizer (15-16-17) was applied once a week. All plants were maintained in close proximity and were subjected to similar environmental conditions.

Chemical Analysis. The two major anacardic acids that comprise 98% or more of the trichome exudate were collected using three different techniques in an attempt to determine whether (1) susceptible plants do not synthesize anacardic acids, or (2) susceptible plants synthesize anacardic acids but are unable

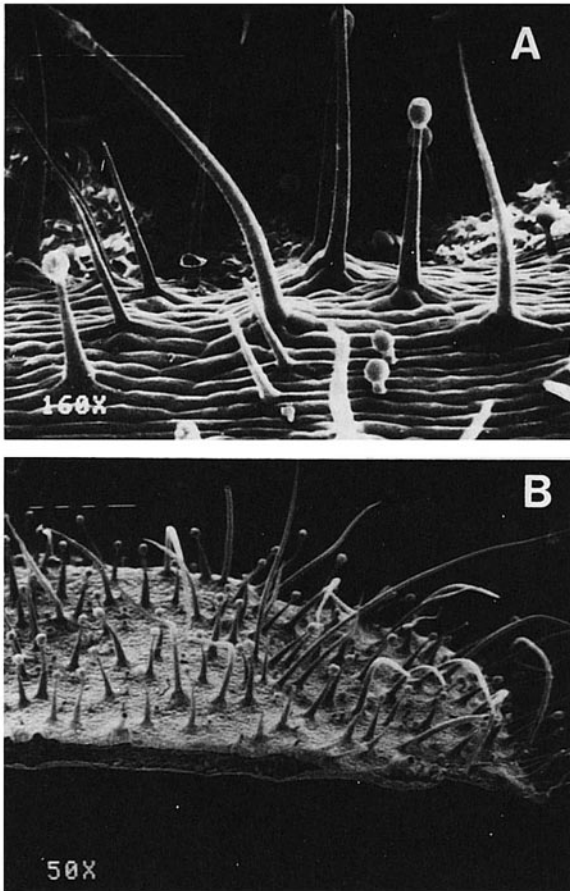


FIG. 1. Scanning electron micrographs of trichomes of geranium (*Pelargonium xhortorum*): (A) upper leaf surface, 160 \times ; (B) calyx surface, 50 \times .

to translocate them to the trichome surface from their site of synthesis, thus reducing the efficiency of the system.

The first method of collection was designed to provide an estimate on a per trichome basis of the quantity of anacardic acids present on and in the trichomes of three of the geranium lines. A Pasteur pipet drawn to a fine diameter, just large enough to fit over a glandular trichome, was immersed in CH_2Cl_2 and the tip filled by capillary action. Using a binocular dissecting microscope, the tip containing the CH_2Cl_2 was placed over individual tall glandular trichomes and the solvent was allowed to extract the trichome for 10 sec. After 10 tri-

chomes had been extracted in this manner, the contents were blown into a collection vial. The entire process was repeated 50 times for a total of 500 trichomes extracted. The 500 trichomes extracted came from four leaves from four separate plants for each inbred line. Resistant line 71-17-7, susceptible line 17-10-1, and the line classified by Winner as resistant but of questionable status (71-18-6) were analyzed by this technique. After collection, the extract was evaporated to dryness under N_2 and redissolved in 50 μ l of CH_2Cl_2 containing 50.6 mg/liter octacosane as an internal standard. A 2- μ l aliquot of this final solution was then analyzed by gas chromatography using a 6-ft mixed phase SP2250/SP2401 (1.5%/1.95%) column and N_2 carrier (66.0 ml/min) at 235°C isothermal conditions. Peak areas were determined using a Hewlett Packard 3390-A integrator. A standard curve using known concentrations of anacardic acids was generated to enable approximate concentrations of anacardic acids per trichome to be determined.

The second method of anacardic acid collection is a modification of a technique described by Gerhold et al. (1984), intended to collect only that exudate on the external trichome surface by physical adhesion to the glass slide. Five plants from each of the five lines discussed earlier were included in this technique for a total of 25 plants. Three leaves collected from the third node from the apical shoot meristem were used for each analysis. Two clean glass microscope slides were pressed firmly against the leaf, sandwiching the leaf between them. The exudate was then washed from the slide into a collection vial using three 0.5-ml rinses of CH_2Cl_2 . The entire leaf surface was covered twice using this procedure. The solvent was then evaporated under N_2 , and the extract was redissolved in 200 μ l of CH_2Cl_2 containing the octacosane internal standard (50.6 mg/liter). The extract was analyzed on the gas chromatograph as previously described, immediately after the addition of the solvent. This technique was intended to collect only exudate from the external trichome surface by physical adhesion to the glass slide. This method, although not quantitative, provides a reasonable estimate of the amount of anacardic acid exuded to the trichome surface. The entire analysis was repeated for all 25 plants on two separate dates.

The third method of collection was a whole leaf extraction in which two leaves from each plant were immersed for 20 min in 10 ml of CH_2Cl_2 . The five individually labeled plants of each inbred line were analyzed for a total of 25 plants. The total fresh weight of two healthy leaves from the third node from the apical shoot meristem was determined. Preparatory experiments have shown that approximately 90% of the anacardic acids are removed in this simple immersion technique when compared to a more thorough extraction in which the tissue is homogenized. This recovery was deemed adequate since we were more concerned with relative differences between plant lines than absolute quantification. Following extraction, the solvent was evaporated to dryness

under N_2 and the sample was redissolved in 0.5 ml of CH_2Cl_2 containing octacosane as an internal standard. The sample was then analyzed by gas chromatography as described for the first collection technique.

Correlation analysis between leaf weight (an estimate of leaf "size") vs. total anacardic acid content within lines showed no significant correlation between these parameters so that leaf weight was ignored in comparisons between lines (see Discussion). The use of third-node leaves of similar size should minimize physiological differences based on leaf size or age. The only correction applied to the individually measured peak areas for the two anacardic acids was to divide each area by the area of the internal standard to correct for differences in the extract volume injected. This procedure was performed on all 25 plants in replicate one month apart, to obtain more statistically reliable results and isolate any transient environmental impacts on leaf chemistry.

Data from the second and third extraction procedures were analyzed statistically using a hierarchical design one-way analysis of variance to determine if differences between lines were greater than differences within a line. Different plants within a line was the subordinate factor, and geranium lines was the treatment factor. Means for resistant vs. susceptible, 17-7 vs. 17-1, and 10-1 vs. 15-4 vs. 18-6 were then compared using a priori methods for partitioning of the between group sums of squares (Sokal and Rohlf, 1969).

Morphological Analysis. The same five geranium lines quantitatively analyzed for anacardic acid were also analyzed morphologically for trichome densities. The densities of all four of these trichome types on the leaf surface were determined using scanning electron microscopy. While the short glandular trichomes possess a globular head, there is at present no evidence of a secretory function for this trichome type in geranium. Nevertheless, in this paper we will refer to them as short "glandular" trichomes based solely on appearance. Leaf samples of each inbred line were removed at random from geraniums grown together under uniform greenhouse conditions. Leaves were collected from the second and the fifth nodes, to permit trichome density comparisons between young expanding leaves and older more mature leaves. Square sections (3×3 mm) were removed in equal numbers from the area near the petiole, the middle leaf area, and the area near the leaf edge of each geranium line. A minimum of 10 (usually more) leaf sections from three leaves from three separate plants were observed for each leaf region for both the upper and lower leaf surfaces. This enabled comparisons between upper and lower leaf surfaces to be made and ensured an adequate representation of all areas of the leaf in the analysis.

The leaf sections were fixed in a 1:1:18 (formalin-acetic acid-ethanol) solution and were dehydrated in an ethanol series (from 20% to absolute). The following day the material was critical point dried using a Polaron E 3000 instrument and gold sputtered with a IDI-PS2 sputter coater. Observations were made and photographs taken with a model ISI-60 scanning electron microscope.

The numbers of trichomes of each type occurring on each section were determined and recorded. In all, data were recorded from approximately 900 leaf sections from six inbred lines including the five lines analyzed chemically.

Before statistical analysis, trichome counts were transformed using the square root transformation, $Y^* = \sqrt{0.5 + Y}$, to obtain a more normal distribution (Sokal and Rohlf, 1969). The transformed data were then analyzed using a two-way analysis of variance. Two separate analyses were performed, one in which the factors in the analysis were inbred lines and leaf age (second and fifth nodes) and the other in which the factors were geranium inbred line and leaf surface (upper or lower). In these analyses the data from separate leaf regions were pooled and differences in trichome densities between them were not analyzed statistically. The densities for each trichome type were analyzed individually. Densities of trichomes are reported as retransformed means from the above analysis and therefore exhibit asymmetrical confidence intervals (Sokal and Rohlf, 1969).

For each two-way analysis, the levels of the factor not included in the analysis were analyzed as separate data sets. For instance, in the analysis of variety \times age, two separate analyses were performed, one for the upper leaf surface and one for the lower, to reduce the variability from influences not directly pertinent to the factors of interest.

Since each analysis was performed separately on the four trichome types, a total of 16 sets of two-way analyses were performed. In addition to the above analysis, a stepwise discriminant analysis using the BMDP-7M statistical program package was performed. For this analysis the two resistant lines and the two susceptible lines were used to create the discriminant function between "susceptible" and "resistant" groups. Again these data were grouped using four different combinations of the age factor (second or fifth node) and leaf surface factor (upper or lower) to provide a more homogeneous data set for the analysis. Thus, four analyses of this type were performed. The four variables (trichome densities) were allowed to enter or were removed from the discriminant classification function based upon F statistic values. Variables were entered when $F > 4.0$ and removed if $F < 3.996$. The discriminant functions created by these analyses were then used to classify the 18-6 line which, although classified as resistant by Winner, seemed to resemble the susceptible plants more closely in some respects.

RESULTS AND DISCUSSION

Chemical Quantification. The means and standard errors of the quantification of anacardic acids isolated using the three different extraction techniques are presented in Figure 2.

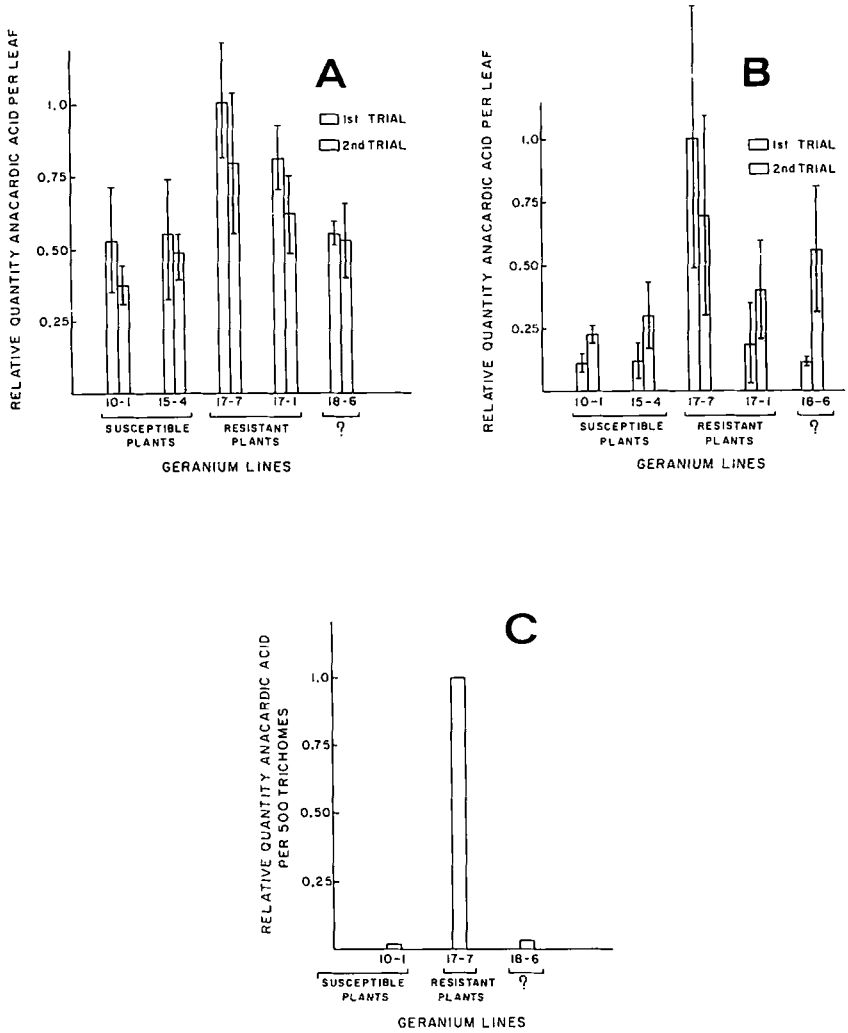


Fig. 2. Graphs of the quantification of anacardic acids from the resistant and susceptible geraniums: (A) whole leaf extract; (B) leaf press method; (C) capillary solvent extraction of individual trichomes.

The first collection technique, that of extracting individual trichomes, showed the most dramatic differences between the susceptible and resistant lines (Figure 2C); the resistant 71-17-7 trichome extract, possessed over 50 times the amount of both anacardic acids found in the susceptible 71-10-1 line extracts.

Using a standard curve based on HPLC purified anacardic acids, resistant line 71-17-7 is estimated to contain 0.11 μg per trichome.

The microscope slide collection technique was employed to estimate the trichome exudate present on the trichome exterior. As shown in Figure 2A and 2B, the differences between geranium lines for anacardic acids collected using this technique were far more dramatic than those shown in whole leaf extracts. The 71-17-7 line exhibited values close to 10 times those seen in the susceptible lines.

From the results of the whole leaf extractions (Figure 2A), it is clear that anacardic acids are present in the susceptible plants, but are less abundant. Analysis of variance of the quantification of anacardic acids showed significant differences between the five inbred lines for whole leaf extractions and microscope slide collection, the resistant plant lines having significantly higher mean concentrations of both compounds (Table 1). A priori decomposition of the between-line sum of squares shows that 44% of the leaf press between group sum of squares and 85% of that for whole leaf extraction are contributed by the resistant vs. susceptible comparison. While the most mite-resistant line (Stark,

TABLE 1. ANALYSIS OF VARIANCE

Source of variation	<i>df</i>	SS	MS	<i>F</i> _s
Leaf press quantification of anacardic acids				
Among plant lines	4	0.375	0.094	11.73 ^a
Resist. vs. Suscept.	1	0.165	0.165	20.62 ^a
17-1 vs. 17-7	1	0.190	0.190	23.75 ^a
10-1 vs. 18-6 vs. 15-4	2	0.020	0.010	1.25 ^b
Among plant within lines	20	0.080	0.004	< 1 ^b
Within plants (error)	25	0.216	0.008	
Total	49	0.672		
Whole leaf quantification of anacardic acids				
Among plant lines	4	6.326	1.581	15.14 ^a
Resist. vs. Suscept.	1	5.362	5.362	107.00 ^a
17-1 vs. 17-7	1	0.744	0.744	14.85 ^a
10-1 vs. 15-4 vs. 18-6	2	0.220	0.110	2.19 ^b
Among plants within lines	20	2.089	0.104	2.08 ^b
Within plants (error)	25	1.253	0.050	
Total	49	9.668		

^a*P* < 0.001.

^b*P* > 0.05.

1975), 71-17-7, also possesses the highest concentration of anacardic acids from the whole leaf extraction, this concentration is only about double the concentration of anacardic acids in the susceptible plants. This difference may be biologically significant as far as resistance is concerned, but is probably insufficient to explain the extreme differences in susceptibility exhibited by greenhouse populations containing both susceptible and resistant lines.

The results of these experiments suggest that rather than lacking the ability to synthesize the anacardic acids, as suggested by Gerhold et al. (1984), the susceptible plants may lack some essential element for translocating the compounds to the upper parts of the trichome and/or the trichome exterior from some unknown site of synthesis within the leaf or trichome base. Gerhold et al. used a lighter touch to collect the exudate, which might explain why they did not observe the anacardic acids in susceptible plants. The mechanism of transport of the exudate is unknown at this time. The inability to transport the compounds may result in their buildup at the biosynthetic site and feedback inhibition of anacardic acid synthesis or simply a shift in equilibrium between precursors and anacardic acid products. Thus the somewhat reduced levels of anacardic acids determined from the whole leaf extracts of the susceptible plants could be a secondary effect caused by an inefficient or inactive transport mechanism rather than the actual cause of susceptibility.

Line 71-18-6, which had been designated as resistant by Winner (1975), showed levels of anacardic acids as extracted by all three techniques to be more consistent with the levels in the susceptible plants rather than the resistant ones. It is important to note that line 71-18-6 is very closely related to 71-17-1 and 71-17-7 (which are sibs) and that there is no close relationship between 71-18-6 and either of the susceptible lines. When 71-18-6 was compared with the two susceptible lines using a priori decomposition of the sum of squares, no significant differences were observed (Table 1). Day-to-day observations of this plant line in greenhouse cultures have indicated it is of intermediate character concerning pest resistance. The discriminant function based on tall glandular and tall spine trichome density differences between resistant and susceptible lines indicated 71-18-6 to fall in the "resistant" category.

Trichome Density Quantification. Height distributions of the four trichome types are presented in Table 2. These data illustrate the clear separation of both glandular and nonglandular trichomes into discrete long and short size classes with little overlap. In addition to size, the long and short glandular trichomes may be distinguished by observing the epidermal cells adjacent to the trichome base. Long glandular trichomes possess a group of six cells arrayed in a spoke-like pattern about the trichome base, while the short type possess no such associated epidermal cells (Figure 1A). In addition, the brownish yellow exudate present in the long glandular trichomes from resistant plants is completely lacking in all short glandular trichomes.

TABLE 2. GERANIUM LEAF TRICHOME SIZE DISTRIBUTION

Trichome type	Sample size	Mean height (μm)	Standard error (μm)	Height range (μm)
Short glandular	90	46.22	4.87	30-60
Tall glandular	74	206.89	9.06	100-400
Short spines	61	136.88	4.55	60-200
Tall spines	56	328.57	13.74	220-600

Short Glandular Trichomes. A summary of the results of the two-way ANOVAs is presented in Table 3. There is virtually no correlation between mite resistance and the density of short glandular trichomes (Figure 3A). The two-way ANOVA for plant line \times surface showed no significance for plant line when the second-node leaves were compared. The fifth-node leaves did possess significant differences between lines, but there was no apparent association with resistance since the significance resulted from the high density of short glandular trichomes on 71-17-1, with all other lines having lower and similar densities. The two-way analysis for plant line \times leaf age was divided into two data sets, one for the upper leaf surfaces and one for the lower surfaces, as described earlier, to provide for a more homogeneous data set. This is especially important in this analysis since the surface (upper and lower) has a large impact on short glandular trichome density (Figure 3A).

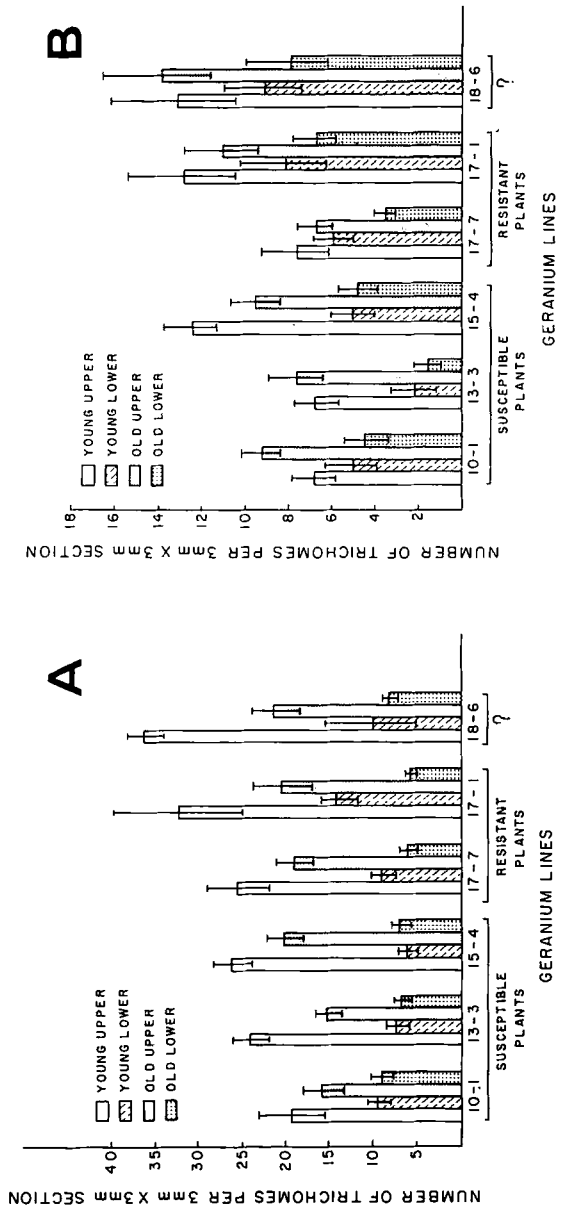
Resistant line 71-17-1 has the greatest density of short glandular trichomes for three of the four leaf surface classes (old upper, old lower, young upper, young lower), but resistant line 71-17-7 has only moderate densities for this trichome type (Figure 3A). Conversely, susceptible line 71-15-4 exhibits high densities of short glandular trichomes on upper leaf surfaces but more moderate densities on the lower surfaces. The fact that upper leaf surfaces possess short glandular trichome densities two to three times that of the lower leaf surfaces and that there is no correlation between degree of resistance and short glandular trichome density indicates these trichomes may not function in the resistance mechanism.

Short Spines. Density data for short spiny trichomes are illustrated in Figure 3B. Again, as with the short glandular trichomes, the ANOVA indicates significant differences in the density of short spiny trichomes among plant lines, but the differences are not related to mite resistance of the plant. As with the short glandular trichomes, resistant line 71-17-1 and susceptible line 71-15-4 exhibit the highest densities, with the other three lines possessing lower, somewhat variable densities. The variety that shows the greatest resistance to mite and aphid attack, 71-17-7, possesses short spiny trichome densities comparable

TABLE 3. SUMMARY OF TWO-WAY FACTORIAL ANOVAS ON TRICHOME DENSITIES^a

Trichome type	Plant line × surface factorial			Plant line × age factorial		
	Source	Leaf age		Source	Upper	Lower
		Young (2nd node)	Old (5th node)			
Short spine	Variety	***	***	Variety	***	***
	Leaf surface	***	***	Leaf age	NS	***
	Interaction	***	***	Interaction	***	NS
Short glandular	Variety	***	NS	Variety	***	***
	Leaf surface	***	***	Leaf age	***	***
	Interaction	borderline	***	Interaction	NS	***
Tall spine	Variety	***	***	Variety	***	***
	Leaf surface	NS	***	Leaf age	borderline	***
	Interaction	***	NS	Interaction	***	NS
Tall glandular	Variety	***	***	Variety	***	***
	Leaf surface	NS	***	Leaf age	borderline	NS
	Interaction	***	NS	Interaction	***	borderline

^a*** $P < 0.001$; NS $P > 0.05$; borderline $0.05 < P < 0.001$.



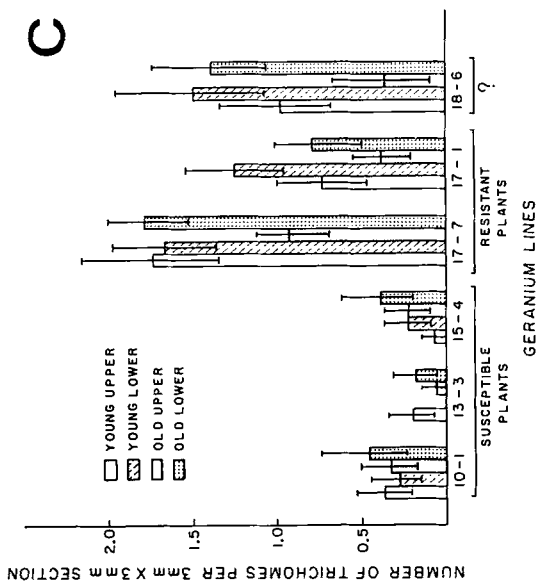
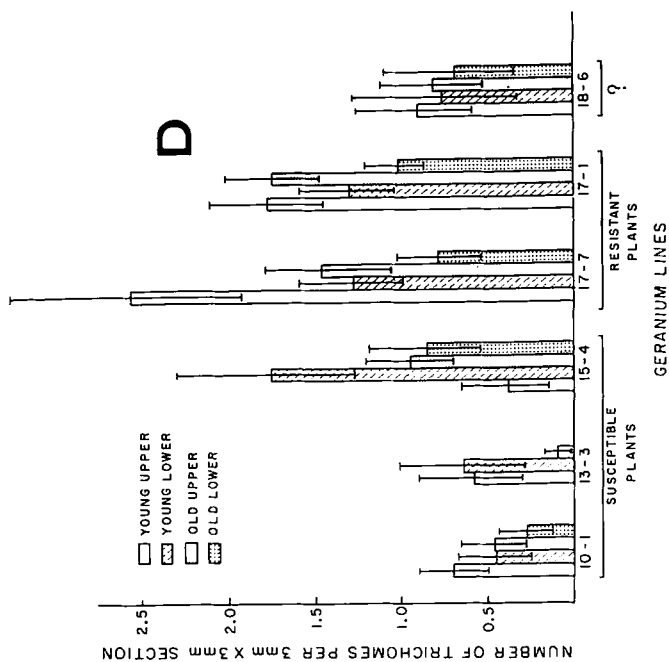


FIG. 3. Distribution of geranium trichomes on resistant and susceptible lines: (A) short glandular trichomes; (B) short spine trichomes; (C) tall glandular trichomes; (D) tall spine trichomes.

to those of the susceptible varieties 71-10-1 and 71-13-3. Likewise, in a distribution similar to that of the short glandular trichomes, although to a somewhat lesser extent, the density of the short spiny trichomes is clearly greatest on the upper leaf surface. While of possible importance in pest resistance, this type of trichome appears not to be the character lacking in our mite- and aphid-susceptible plant lines. If such were the case, one might expect 71-15-4 to be more resistant than it is. Figure 3B illustrates the homogeneous distribution of short spine density between plant lines and the lack of association with mite resistance. With some exceptions, the density of short spines decreases in the older more fully expanded leaves parallel to the decrease shown by the short glandular trichomes. It should not be implied here that the short spines are not a factor in pest resistance. Mites and aphids observed on the leaf surface have difficulty in traversing these spines and in finding acceptable feeding locations on the leaf because of them. It can be stated, however, that they alone are unable to provide resistance to the pests thus far assayed (mites, aphids, whiteflies).

Tall Glandular Trichomes. Density data for this portion of the indumentum is presented in Figure 3C. The trichome density differences between lines are highly statistically significant and closely associated with the resistance phenomenon. The most resistant line, 71-17-7, has by far the highest tall glandular trichome densities, followed by its resistant partner, 71-17-1. The density of tall glandular trichomes on 71-17-7 leaves is three or more times that of the susceptible plants in all four leaf regions analyzed. Figure 3C illustrates the extremely large differences between resistant and susceptible plant lines in terms of tall glandular trichome densities. While exhibiting some variability, the density values determined for each of the plant line \times leaf age subclasses are reasonably consistent. It appears that tall glandular trichome density may be a primary factor in determining degree of mite resistance in (*P. xhortorum*). This is in spite of the fact that the short glandular and short spiny trichomes are nearly 10 times as abundant on the geranium leaf surface. In contrast to the previous two trichome types, the tall glandular trichomes do not exhibit a markedly higher density on the upper leaf surface. There is no consistent trend for differences between upper and lower densities; each of the inbred lines exhibits its own trend, and the differences are not pronounced. Differences between young and old leaves are also not as clear in this trichome type as in the others. The two-way ANOVA for plant line \times leaf age, using data for the lower leaf surface, showed the differences to be nonsignificant, while the differences shown among upper leaf surfaces were of borderline significance. This again is probably more a result of the low tall glandular trichome densities and the resulting possibility for somewhat larger error in estimating true densities. This is supported by microscopic observations that young undeveloped tall glandular trichomes are not present on any but the youngest and most undeveloped geranium leaves, and thus they must decrease in density as the leaf expands and ages. It may be noted that although no formal comparisons were made, the tall glandular tri-

chome densities found on the pedicel and sepal surfaces of the inflorescence are clearly several times those found on leaf surfaces in all plant lines examined. Investing much of its defensive energies in protecting structures responsible for continuation of its line might be advantageous for a plant and a further indication of the importance of the tall glandular trichomes in pest resistance.

Tall Spines. The fourth and final trichome type analyzed for density was the tall spines. These data are presented in Figure 3D. Comparison of tall spiny trichome densities between resistant and susceptible plants indicates a fairly close association between high density and resistance (Figure 3D). Both resistant lines possess densities much higher than two of the three susceptible lines; however, 71-15-4 has relatively high densities equal to those of the resistant lines. If, as suggested by the high densities found on the resistant plants, the tall spiny trichomes do play a role in the resistance phenomenon, they clearly are not sufficient in themselves to provide resistance given the high densities found on susceptible 71-15-4. In all cases, save one, comparing the tall spiny trichome density for a specific line and leaf surface shows the younger surface to be more dense than the older one. Inspection of the data shows that tall spiny trichome densities are similar to the tall glandular trichome densities and well below those of the two short trichome types. With two exceptions (young leaves of 71-15-4 and 71-13-3), the upper leaf surface possesses more tall spines than the lower leaf surface following the general trend of both the short spines and the short glandular trichomes.

Analysis of variance of these data generated in this study showed significant differences for most of the interactions and main effects tested for all four trichomes. There is, however, a distinction between statistically significant and biologically meaningful differences as related to their importance in the resistance mechanism. Such differences must be correlated with the expression of resistance in order to suggest a function within the resistance mechanism.

Microscopic observations of many geranium leaf surfaces have shown, without exception, that even extremely young leaves possess a full complement of trichomes which, because of the unexpanded nature of the leaf, are very dense. This may be an economical way for the plant to protect the leaf most when it is young and with a long useful period of productivity ahead of it; it expends less energy and resources on protection of the leaf as it ages and the period of its usefulness to the plant is decreased.

From the results of this study it may be concluded that there are two factors possessed by mite- and insect-resistant lines of geraniums that are lacking in the susceptible varieties used in this study. Resistant lines have higher densities of tall glandular trichomes and are better able to express the anacardic acids as an exudate on the trichome exterior. Furthermore, we have shown that the susceptible lines, while possessing somewhat less anacardic acid per leaf, are nevertheless able to manufacture the compounds in significant quantities. Consequently, one genetic defect in the susceptible plants could be a malfunctioning

component of the translocation system for the defensive compounds. Plant line 71-18-6 is of special interest for future biochemical and genetic work, since it seems to possess a complement of tall glandular trichomes equal to that of the resistant plants but, at the same time, under some conditions, it is deficient in the expression of anacardic acids as exudate on the trichome surface as are the susceptible plants (unpublished data).

Although the tall glandular trichomes are apparently of primary importance in the resistance mechanism, it seems likely that the spiny trichomes are a necessary component as well. The fact that line 71-17-1 is close to 71-17-7 in degree of mite resistance may be due to the high density of short spines 71-17-1 produces, even though it produces less exudate from the tall glandular trichomes. By restricting movement and forcing a mite, aphid, or other small insect to travel between the long spines, the geranium is able to cause increased contact between the pest and the tall glandular trichome exudate. The spines are undoubtedly "cheaper" for the plant to produce and maintain in terms of energy and nutrient resources than an equivalent number of the glandular trichomes. Thus, the plants can increase the effectiveness of a relatively low density of exudate-producing glandular trichomes by producing higher densities of the metabolically less expensive spiny trichomes.

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CHEMICAL ASSOCIATION IN SYMBIOSIS Sterol Donors in Planthoppers

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Abstract—The role of intracellular symbionts contributing to their host has been investigated in the planthoppers, *Nilaparvata lugens* Stal and *Laodelphax striatellus* Fallen. We have found that the isolated yeastlike symbionts, identified as a member of the genus *Candida*, from the host's egg produce ergosterol when cultured. A comparative study of sterols in the cultured symbionts, the host insects, aposymbiotic host insects, and dietary plants demonstrated that ergosterol produced in the symbiotes is provided to the host insects and possibly transformed in the host insects into cholesterol via 24-methylenecholesterol. The conversion of injected 24-methylenecholesterol- d_3 into cholesterol has been shown in the brown planthopper (*N. lugens*).

Key Words—Biotransformation, cholesterol, 24-methylenecholesterol, ergosterol, brown planthopper, *Nilaparvata lugens* Stal, smaller brown planthopper, *Laodelphax striatellus* Fallen, Homoptera, Fulgoridae, symbiosis, intracellular symbiont.

INTRODUCTION

Most arthropoda, such as insects and crustaceans, require cholesterol, since they are incapable of steroid synthesis *de novo* (Clayton, 1964) and utilize dietary steroids as the source of membrane constituent as well as the precursor of 20-hydroxyecdysone (Svoboda et al., 1975), the common molting hormone. In contrast with most other insects, plant-sucking homopterans, such as planthoppers, aphids, and leafhoppers, can be reared on a chemically defined artificial

diet free of sterols (Noda and Mittler, 1983), an observation suggesting the nutritional role of symbionts harbored (Nasu, 1965) in these insects. However, this is disputed in a recent study on the aphid *Schizaphis graminum* (Campbell and Nes, 1983). The intracellular symbiotic microorganisms contained within the mycetocytes or fat bodies of aphids, leafhoppers, and rice planthoppers are transmitted into the eggs to the next generation via transovarial infection (Noda and Mittler, 1983). The success in isolating yeastlike symbionts of *L. striatellus* and *N. lugens* and maintaining them in permanent culture (Kusumi et al., 1979, 1980; Nasu et al., 1981) has made it feasible to study the biosynthetic capabilities of these symbionts in the culture medium. Preliminary studies with several symbiotic cultures have demonstrated that symbionts play a dual role (Fredenhagen et al., 1987a), i.e., provision of sterols to the host insect and production of antibiotics for the self-defense of hosts (Fredenhagen et al., 1987a,b).

We describe here qualitative and quantitative analysis of the steroids in the cultured symbionts, the host insects, the aposymbiotic host insects, and the dietary plants. The results indicate that the yeastlike symbionts produce ergosterol and provide it to the host as the possible source of 24-methylenecholesterol and cholesterol. This hypothesis was supported by the transformation (35%) of injected 24-methylenecholesterol- d_3 into cholesterol in the brown planthopper (*N. lugens*).

METHODS AND MATERIALS

Insects and Host Plants. The brown planthopper (rice BPH), *N. lugens*, and the smaller brown planthopper, *L. striatellus*, were obtained from the International Rice Research Institute in the Philippines, while the leersia BPH was collected in Indonesia. The insects were reared in plastic cages with their host plants, *Oriza sativa* L. Koshihikari for rice BPH and *Leersia hexandra* for leersia BPH, the plants being grown in potted soil, 5–10 cm high. The cages containing insects and host plants were kept in a greenhouse at 25–28°C, with a 16-hr light and 8-hr dark photoperiod. For a comparative study, both sexes of third- to fourth-instar nymphs, adults, and aposymbiotic insects were used; the third-instar nymphs and adults were maintained at 35°C for three days and then transferred to room temperature, 25°C, and allowed to feed on rice plant for seven days. These aposymbiotic insects have greatly reduced numbers of symbionts and fail in ecdysis. However, dietary administration of cholesterol rather than sitosterol partly overcomes this problem (Noda and Saito, 1979). The leaves and stems of the dietary plants, Koshihikari, and *L. hexandra* were extracted and analyzed, respectively, as described below.

Symbionts. The morphologically and physiologically different yeastlike symbionts were isolated from the eggs (Kusumi et al., 1979); i.e., Ls-1 and

Ls-2 were from *L. striatellus* and N1-1 and N1-2 were from *N. lugens*. After pasteurization of the surface, the eggs were homogenized in saline and cultured on Grace's agar plate. The symbionts thus isolated were cultured in 1 liter of sterol-free Burkholder medium for four weeks at 30°C. The work-up of the culture broths was as described for the isolation of the sterols from insects (see below). These yeastlike symbionts were identified as a member of the genus *Candida* (Amano, unpublished¹).

Biotransformation. Brown planthoppers *N. lugens*, of third- and fourth-instar nymphs were injected (between the midcoxa and metasternum) with a glass microsyringe with deuterium-labeled 24-methylenecholesterol (see below) emulsified in 20% glycerol and 0.2% Emulgen 913 in water, at a concentration of 2mg/100 μ l (about 0.001 μ l/insect). The injected insects were transferred to cages with rice plants and sacrificed on day 3 or 7 by chilling in a -20°C freezer to see the relative rate of biotransformation.

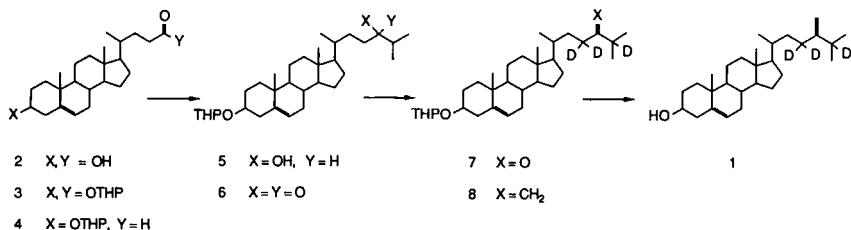
Isolation of Steroids. Using a Tenbrock grinder 130-200 insects were homogenized in 10 ml chloroform-methanol (2:1) and sonicated for 1 hr. The suspension was centrifuged at 3000 rpm for 5 min, and the organic layer was evaporated to dryness. The residue was then extracted with 40 ml hexane-methanol (1:1) and divided into two fractions. The upper layer, containing free sterols and their esters, was hydrolyzed (90°C, 3 hr) with 0.5 ml 10% methanolic potassium hydroxide in 5 ml water. The lower layer, containing steroidal glycosides, was treated with 5% methanolic hydrogen chloride at 90°C for 3 hr. After work-up of each hydrolysate, the combined free sterol fraction was purified on a silica gel TLC (Kieselgel 60 F254 DC-Fertigplatten Art. 5744 and HPTLC-Fertigplatten Art. 5628, Merck) using ether-hexane (1:1) and the R_f region corresponding to sterols was removed and eluted with chloroform-methanol (2:1). After evaporation of the solvent, the residue was silylated in the usual manner using pyridine, hexamethyldisilazane, and trimethylchlorosilane (2:1:1 v/v). The TMS-derivatized sterols were dissolved in 20 μ l chloroform and 5- μ l aliquots of this solution were analyzed using GC-MS. The TMS-derivatized sterols from the control insects of the same colony reared under identical conditions were used for comparative purposes in the insect biotransformation.

Analysis. Sterols were analyzed with a Hitachi M-80 GC-MS spectrometer equipped with a OV-1 packed column (3 mm \times 1 m). GC conditions were as follows: oven temperature 175-250°C (2°C/min), He gas flow rate 4.7 ml/sec. The Hitachi mass spectrometer was linked to a model M-003 data processing system. The transformation of [23,25-d₃]-24-methylenecholesterol to cholesterol in the insects was measured by intensity enhancement of the M+3 isotope

¹ Referring to Kreger-van Rij (1984), we identified both yeast strains, Ls-1 and NL-2 as a member of the genus *Candida*; Kreger-van Rij, N.J.W. (ed.), 1984. The Yeasts, A Taxonomic Study. XVI + 1082 pp., Elsevier, Amsterdam.

peak due to deuterium incorporation. The dispersion of the M+3 (m/z 461) isotope peak intensity of the standard TMS cholesterol (or recovered TMS cholesterol from insects) at $1 \mu\text{g}/5 \mu\text{l}$ chloroform was maximally 3.5% in 12 repeated runs of 10–20 scans, the m/z 458 M^+ peak being adjusted to 100%.

Chemicals. Sterol standards and reagents were purchased from Nakarai Chemicals, Ltd. and Tokyo Kasei Kogyo Co., Ltd. Emulgen 913 (biological detergent) was a gift from Kao Co., Ltd.



SCHEME 1. Synthetic scheme of 24-methylenecholesterol- d_3 .

Synthesis. The deuterium-labeled [23,25- d_3]-24-methylenecholesterol **1** was synthesized in seven steps from 3 β -hydroxy- Δ^5 -choleonic acid **2** (Scheme 1). The THP-ester **3** (**2** and 3 equiv. of dihydropyran in benzene with pTSA at room temperature under N_2 for 18 hr, 76%) was transformed into the corresponding aldehyde **4**: 3 equiv. of diisobutylaluminum hydride in 1 M hexane solution in toluene at -78°C for 15 min under N_2 (Zakharkin and Khorlina, 1962), quenched with 50% aq. MeOH at -78°C for 30 min and chromatography on alumina, hexane–ether, 85:15, 73%. Grignard coupling of **4** with isopropyl magnesium iodide gave **5** (3 equiv. in diethylether at 25°C for 1 hr followed by usual treatment), which was oxidized to ketosteroid **6** (pyridinium chlorochromate in dichloromethane under N_2 at 25°C for 30 min).

The ketosteroid **6** was transformed into the desired product **1**, overall 80%, by the following sequence: (1) Deuteration of ketosteroid (41.5 mg of **6** in 4 ml methanol- d_4 , 0.5 mg of deuterium oxide and 3 mg of anhydrous sodium carbonate at 70°C for 20 min, evaporated to dryness and the cycle repeated four times; Nolin and Jones, 1952); (2) Wittig coupling to give the 24-methylene steroid **8** (deuterated ketosteroid **7** with 2 equiv. of triphenylmethylphosphorane in ether under N_2 at 25°C for 15 min; Newman, 1960); (3) hydrolysis (1 N HCl in methanol for 1 hr at 25°C , neutralization with sat. aq. sodium bicarbonate, extraction and flash chromatography on Kieselgel 60 Art 9385, hexane–ethyl acetate). The synthetic product **1** was identified by the NMR spectrum recorded on a Nicolet NT-360 spectrometer, $\delta(\text{CDCl}_3)$: 5.35 (1H, m, 6-H), 4.71 and 4.66 (2H, dd, $J = 2, 28$ Hz, 24-H), 3.48–3.58 (1H, m, 3-H); m/z

401, $C_{28}H_{43}D_3O$: (M^+), (26%); $386(M-CH_3)^+$, (39%); $314 (M-C_6H_9D_3)^+$, (100%). The percentages of d_3 (m/z 401), d_2 (m/z 400), and d_1 (m/z 399) of the deuterated 24-methylenecholesterol after subtraction of natural abundance ^{13}C isotope were 54.0, 42.6, and 5.0% respectively.

RESULTS AND DISCUSSION

Sterols from Cultured Intracellular Symbionts. Ergosterol was the only sterol found in the culture broth (1 liter) of Ls-1 and Ls-2. Mixed cultures of Ls-1 and Ls-2 or addition of mevalonic acid (5 mg/l) did not change the products. No sterols could be detected, however, from the cultured medium of symbionts NI-1 and NI-2. Instead, a series of compounds i.e., lanosterol, 24-methylenelanosterol, dihydroergosterol, and ergosterol (major), suggestive of an intracellular biosynthetic buildup of ergosterol from mevalonic acid was detected in the cells of NI-1 and NI-2. The cells of Ls-1 and Ls-2 also contained the same set of compounds, although in much smaller quantities. The above result indicates that ergosterol was found in intact *N. lugens* but only a trace amount in *L. striatellus* (see below). Detection of 24-methylenecholesterol in *L. striatellus* and *N. lugens* may demonstrate differences in the mode in which the symbiont-synthesized sterol was made available to the host, an observation in agreement with the hypothesis that different symbionts fulfill different physiological roles (Griffiths and Beck, 1975; Hinde, 1971).

Sterols in Host Insects. In both rice brown planthopper (BPH) (*N. lugens*) and leersia BPH, the sterols consisted of cholesterol, 24-methylenecholesterol, and ergosterol (Figure 1). It is noted that the relative content of 24-methylenecholesterol is greater in adults than in nymphs. In both adult and nymph of aposymbiotic insects seven days after heat treatment, the amounts of ergosterol and 24-methylenecholesterol relative to cholesterol, as well as the total amount of sterols, were reduced drastically. On the other hand, in the smaller brown planthopper, *L. striatellus*, a small amount of sitosterol was found. This result is in agreement with a previous report (Noda and Saito, 1979). The ratio of nonconverted dietary steroids to insect steroids was rather small. Approximately 4–8 mg of each sterol was detectable in each of the isolated samples from 130 insects by the mass chromatographic GC-MS of the TMS derivatives. The body cholesterol content of the apo-insects was reduced to $\frac{1}{10}$ of that of the control, in agreement with the previous studies (Noda and Saito, 1979).

Sterols in Host Plants vs. Rice BPH and Leersia BPH. Sterols of the rice plant Koshihikari and *L. hexandra* showed that the former contained sitosterol, stigmaterol, campesterol and their respective esters, while the latter contained sitosterol, stigmaterol, campesterol, cholesterol, and their respective esters and

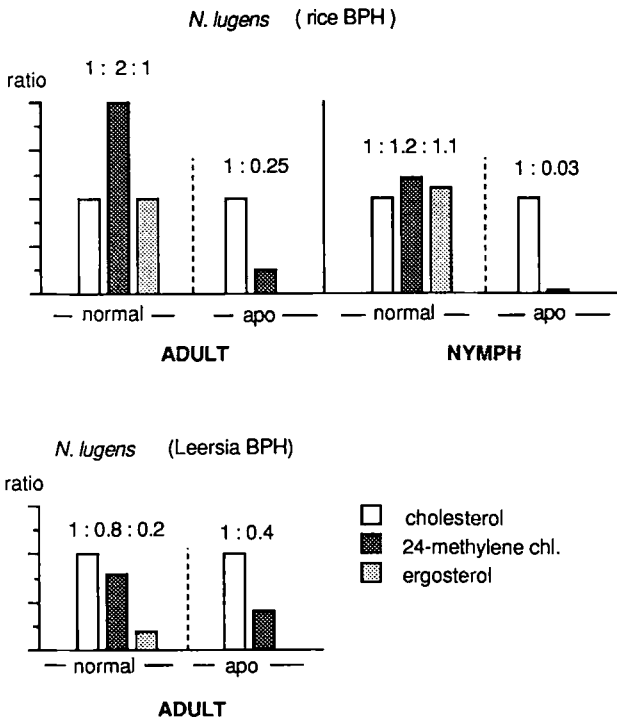


FIG. 1. Sterol constituents in combined sterol fraction from normal and aposymbiotic *N. lugens* (rice BPH and leersia BPH); relative contents of sterols are normalized to cholesterol.

glycosides. The presence of cholesterol in *L. hexandra* may account for the higher ratio of cholesterol in leersia BPH (Figure 1). Thus, the possibility that ergosterol originates from the food chain can be ruled out.

Biotransformation of 24-Methylenecholesterol-d₃ to Cholesterol in BPH.

In vivo conversion was demonstrated upon administration of labeled compound emulsified in glycerin and Emulgen 913® to BPH. The cholesterol-d₃ was found in insects harvested on both days 3 or 7 after injection. On the basis of the injected 24-methylenecholesterol-d₃, 54%, the cholesterol-d₃ recovered from insects was found to be 13 and 19% upon GC-MS (after subtraction of the $\pm 3.5\%$ natural abundance isotope effect, see above), corresponding to conversions of 24 and 35%, respectively. Dealkylation of the 24-methylenecholesterol to cholesterol in BPH was thus proven, the conversion of which was shown previously in *Manduca sexta* (Thompson et al., 1973). The high level of this conversion in the insects demonstrates the utilization of 24-methylenecholesterol as the main source of cholesterol. Although the transformation of ergos-

terol to 24-methylenecholesterol and sitosterol to cholesterol has as yet not been proven quantitatively, comparative study among insects may indicate 24-methylenecholesterol is accumulated after transformation. The conspicuous decrease of 24-methylenecholesterol in the apo-insects may suggest the fast turnover of the ergosterol supplied by the symbionts and not of campesterol from the host plants. Ergosterol is the obligatory nutrient in the ambrosia beetle (*Xyleborus ferrugineus*), which carries endosymbiotic fungi (Rao et al., 1983).

The hypothesis that the sterols are synthesized and supplied, at least partly, by their associated symbionts has been elucidated in the present studies. It has previously been reported that the prokaryotic symbionts in the pea aphids (*Neomyzus circumflexus* and *Acrythosiphon pisum*) synthesize sterols (Ehrhardt, 1968; Houk et al., 1976) in agreement with their possible role as a steroid supplier. However, Campbell et al. (1983) reported that dietary [2-¹⁴C]mevalonic acid was not converted to steroids in the aphid but dietary [4-¹⁴C]sitosterol was converted to cholesterol. Therefore, reinvestigation using isolated symbiont cultures is desirable, because bacteria and mycoplasmal prokaryotes generally lack the ability of steroid synthesis (Nes and Nes, 1980).

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ADDITIONAL SOURCES OF DISTURBANCE PHEROMONE AFFECTING THE CRAYFISH *Orconectes virilis*

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Abstract—The responses of individually housed crayfish (*Orconectes virilis*) were recorded during introduction of water from aquaria containing disturbed or undisturbed animals. When female *O. virilis* were disturbed, form II males assumed low-level alert postures significantly more than when those females were undisturbed. Similar results were obtained when the source animals were female *Cambarus diogenes*, a sympatric crayfish. When the newt *Notophthalmus viridescens* was disturbed, the crayfish responses were strong but resembled stress behavior. The responses by crayfish to disturbed vs. undisturbed catfish (*Ictalurus natalis*) were not significantly different.

Key Words—Pheromone, disturbance, crayfish, *Orconectes virilis*, cross-phyletic communication, stress, female crayfish.

INTRODUCTION

The detection of stressed conspecific individuals has been reported in earthworms (Ressler et al., 1968), several types of molluscs (Landauer and Chapnick, 1981; Sleeper et al., 1980), insects (Levinson et al., 1974), and mammals (Valenta and Rigby, 1968). The responses of crayfish to a chemical or chemicals given off by stressed (but not injured) conspecific individuals have been used to demonstrate the existence of disturbance pheromones (Hazlett, 1985a). Observations (Hazlett, 1985b) have indicated that these pheromones are neither specific to a particular form of stress that a crayfish experiences nor are they

species specific. Individuals of *Orconectes virilis* respond to water containing stressed males of *O. virilis*, *O. rusticus*, or *O. propinquus* (Hazlett, 1985b).

Recent work has indicated a number of cases in which chemical communication occurs across phyletic lines. This often is observed in the form of predator detection (Scrimshaw and Kerfoot, 1987; Petranka et al., 1987; Werner, personal communication) and can have both behavioral and morphophysiological consequences for prey animals. The experiments reported herein were designed to look for chemical communication across major phyletic lines as well as to examine the question of release of disturbance pheromones by female crayfish.

METHODS AND MATERIALS

Experiments were conducted at the University of Michigan Biological Station near Pellston, Michigan, during July and August 1986. Methods were identical to those described earlier (Hazlett, 1985a, b). The crayfish tested were all individuals of *Orconectes virilis* collected from either Douglas Lake or the Maple River. Test individuals were housed in individual 10-gallon aquaria that were visually isolated. Each test crayfish was provided with a rock burrow and was tested with just one type of water on any given day. Water was introduced via a peristaltic pump at the rate of 20 ml/min for a test period of 8 min.

The sources of water introduced to the test individuals of *O. virilis* were aquaria that had been filled partially with untreated, well-aerated water from Douglas Lake and in which one or more individuals of the source animals had been living for two to four days. The types of source animals were two species of crayfish and two species of aquatic vertebrates: female crayfish in a different genus (*Cambarus diogenes*), female crayfish of the same species (*O. virilis*), members of a school of young yellow bullhead catfish (*Ictalurus natalis*) that had been collected in Douglas Lake near Sedge Point, and two individuals of the aquatic phase of the red-spotted newt (*Notophthalmus viridescens*) collected in East Point Pond near Douglas Lake. The two latter species were chosen on the bases of being taxonomically unrelated to crayfish, not common predators of adult crayfish, and cooccurring with the test species.

For the tests when the source animals were female *O. virilis*, all of the test crayfish were male *O. virilis*, which were form II, the sexually inactive form of cambarine crayfish (Word and Hobbs, 1958). In the other tests, both form I males and female *O. virilis* were utilized.

For each type of source animal, water was introduced to resting, undisturbed test animals under two conditions: (1) when the source animals appeared quiet and undisturbed and (2) when the source animals were disturbed by a simulated predator (chased by an aquarium net). All statistical tests compared

the responses shown during introduction of water from the disturbed vs. undisturbed condition for a given type of source animal.

The behavior patterns recorded have been described earlier (Hazlett, 1985a, b) and involved three positions (raised, neutral, lowered) of three body parts (chelipeds, cephalothorax, abdomen). Earlier work showed that the primary response by crayfish to disturbance pheromones was assumption of a neutral posture. Therefore the data compared in this study are the number of seconds spent by test crayfish with any body part in the neutral position. Because a number of the comparisons involved data sets with unequal variance, the non-parametric median test was used for statistical comparisons. Not all the test animals were used with both disturbed and undisturbed water, thus paired statistical tests were not used.

RESULTS

The reactions of test crayfish during the introduction of water from different sources are summarized in Table 1. The strongest difference in behavior patterns shown by individuals of *O. virilis* to water from disturbed vs. undisturbed animals was in the case of the salamander *Notophthalmus viridescens*. The crayfish showed no response (remained in the lowered posture typical of resting animals) when water containing undisturbed newts was introduced. When the newts were disturbed, the reactions were quite rapid and unusual. Although the positions assumed by the crayfish were categorized as "neutral," the postures of the chelipeds and walking legs were not the same as those exhibited during introduction of water from other stressed animals (Hazlett, 1985b). The angles between limb segments were unusual and could best be described as contorted. Rather than appearing to be on "low-level alert" (Hazlett, 1985b), the crayfish behaved as if in a distressed state themselves.

TABLE 1. AVERAGE NUMBER OF SECONDS DURING OBSERVATION PERIODS SPENT BY INDIVIDUALS OF *O. virilis* WITH ONE OR MORE PARTS IN NEUTRAL POSITION WHEN WATER FROM VARIOUS SOURCES WAS INTRODUCED^a

Source of water	N	Mean No. seconds		P from median test
		Undisturbed	Disturbed	
<i>Notophthalmus</i>	14	0	196	0.0003
<i>Ictalurus</i>	36	61	116	0.1904
<i>Cambarus</i>	14	41	104	0.0285
<i>O. virilis</i>	23	52	111	0.0291

^aN represents the total number of tests with a particular source.

The responses shown to disturbed and undisturbed bullhead catfish were not significantly different from one another (Table 1). While the crayfish tended to assume neutral postures more frequently when water from disturbed catfish was introduced, the difference was not significant.

In the case of water from disturbed and undisturbed female crayfish of the species *Cambarus diogenes*, the responses shown by individuals of *Orconectes virilis* were significantly different (Table 1). The crayfish assumed the neutral posture much more frequently when water from an aquarium containing disturbed *C. diogenes* was introduced. In a similar fashion, water from female conspecifics elicited different responses on the part of form II male *O. virilis*, depending upon the state of the female crayfish (Table 1). Form II males assumed one of the neutral postures for more of the observation period during the introduction of water from disturbed female conspecific individuals compared to undisturbed individuals.

DISCUSSION

The observation that crayfish of one species (*Orconectes virilis*) react to chemicals from a variety of species of disturbed animals would seem to strengthen the idea (Hazlett, 1985b) that the chemical(s) involved is not very specific. Production of a chemical or class of chemicals that induces low-level alert in totally unrelated individuals points toward the communication resulting from the detection of a chemical that occurs simply from increased activity on the part of another animal. Neither the conditions leading to the increase in activity nor the type of animal involved appear to be critical. While more experiments with crayfish as recipients are needed to test the generality of this suggestion, what may be of more widespread importance is the possibility that whole communities of aquatic animals could cue in on potentially detrimental situations by responding to (the chemical results of) a disturbance to any member of the community.

It was noted earlier (Hazlett, 1985b) that the lack of a clear response by male crayfish to disturbed female crayfish was puzzling and perhaps the result of confounded chemical messages. That is, introduction of water from disturbed female conspecifics could produce stimulation of males with both sex pheromones (Hazlett, 1985a) and disturbance pheromones. Form II male crayfish are said to be sexually inactive, thus they should be less "confused" by reception of any female sex pheromone. The results of this study indicate that when the chemical message channel is simplified (by use of form II males), the production of disturbance pheromones by females is demonstrable. This was also shown by the response differential shown to waters from disturbed and undisturbed females of the genus *Cambarus*. While there appears to be some cross-species detection of sex pheromones within the genus *Orconectes* (Tierney and

Dunham, 1982), the results reported here would indicate little confusion via sex pheromones across genera.

The responses of the crayfish to water from disturbed newts appeared qualitatively different from the responses to other disturbed animals. The newts may have been giving off a toxin (Pough, 1971), rather than a pheromone in the narrow sense. The lack of a clear differential in response to bullhead catfish by crayfish was a bit surprising given the established response by catfish to disturbance (Al-Hassan et al., 1985; Todd et al., 1967). While it is possible that the "undisturbed" catfish in these tests were not really undisturbed, the demonstration of cross-phyta detection of a nontoxic disturbance chemical remains for future work. It is crucial for such tests that laboratory conditions allow maintenance and observation of undisturbed individuals of both the recipient and source species.

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DIFFERENTIAL EFFECT OF TOMATINE AND ITS
ALLEVIATION BY CHOLESTEROL ON LARVAL
GROWTH AND EFFICIENCY OF FOOD
UTILIZATION IN *Heliothis zea*
AND *Spodoptera exigua*

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Abstract—The effect of tomatine on larval growth of *Heliothis zea* and *Spodoptera exigua* was assessed by rearing larvae on diets with different concentrations of the chemical added. When reared from neonates, linear dose-response relationships were obtained for both species, with *S. exigua* being three times more sensitive to tomatine than *H. zea*. Tomatine toxicity was completely alleviated in *H. zea* by the addition of equimolar cholesterol into the diet; however, in *S. exigua* some toxicity was maintained. Larvae of *S. exigua* that were started on control diet were insensitive to tomatine after five days; larvae started on diet with an EC_{50} of tomatine and then switched to control diet after five days failed to recover from toxicosis. Larval growth of *H. zea*, on the other hand, was affected both at the neonate and third-instar stage, but normal growth resumed when the larvae were transferred to control diet. Tomatine had little or no effect on food consumption, assimilation, or dietary utilization of the food by third-instar larvae of *S. exigua*, except at a concentration 10 times the EC_{50} . In contrast, the efficiency of food utilization of *H. zea* larvae decreased with increasing tomatine concentrations. Assimilation of the food tended to increase, although not significantly, as tomatine levels increased. Food consumption of *H. zea* larvae also increased when the tomatine concentration was greater than an EC_{50} . The addition of equimolar cholesterol to diets with an EC_{50} of tomatine restored weight gain and nutritional indices values to control values. These results are related to the utility of using tomatine in host-plant resistance programs.

Key Words—Lepidoptera, Noctuidae, *Heliothis zea*, *Spodoptera exigua*, *Lycopersicon esculentum*, tomato, saponin, glycoalkaloid, tomatine, cholesterol, developmental sensitivity, nutritional indices, allelochemical interactions, host-plant resistance.

INTRODUCTION

Saponins from a variety of plants have been implicated as bases of constitutive resistance to attack by insects. Saponins from alfalfa seed have been shown to inhibit growth of *Tribolium castaneum* (Herbst) larvae (Shany et al., 1970a,b). Growth reduction is mainly due to the aglycone moiety, with the sapogenin medicagenic acid being most toxic. Applebaum et al. (1965, 1969) showed that the developmental incompatibility of the bruchid *Callosobruchus chinensis* L. with soybeans was at least partly due to the presence of soybean saponins. In contrast to *T. castaneum*, toxicity to *C. chinensis* was the result of the larvae being unable to hydrolyze the saponins to their component free sapogenins and sugars, which individually or as a mixture were nontoxic. Triterpene glycosides in organ pipe cactus inhibit the maturation of *Drosophila nigrospiracula* Patterson and Wheeler larvae (Kircher, 1972). Glycoalkaloids from potato affect larval feeding of the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Sturckow and Low, 1961; Sinden et al., 1980), as well as nymphal survival and duration of settling of the potato leafhopper *Empoasca fabae* (Harris) (Raman et al., 1979). The glycoalkaloid tomatine from tomatoes has been shown to inhibit nymphal growth of the two-striped grasshopper *Melanopus bivittatus* (Say) (Harley and Thorsteinson, 1967) and larval growth of the tomato fruitworm *Heliothis zea* (Boddie) (Isman and Duffey, 1982). Campbell and Duffey (1979) also found that tomatine could reduce pupal eclosion, adult longevity, and cause developmental malformations in the ichneumonid wasp *Hyposoter exiguae* (Viereck) when added to the diet of its host.

In this study, we compare the relative toxicity of tomatine to two herbivorous pests of tomatoes, *H. zea* and *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae), and the potential for the alleviation of tomatine toxicity by cholesterol as evidenced before in the wasp *H. exiguae* (Campbell and Duffey, 1981). Tomatine has been implicated as a factor of resistance in the tomato plant to *H. zea* (Juvik and Stevens, 1982). Our study emphasizes that a more effective utilization of plant natural products such as tomatine as a basis of host-plant resistance (HPR) would arise if we better understood some pertinent facts about the toxicity of these chemicals, in terms of (1) the sensitivity of target and nontarget insects to such chemicals, (2) larval feeding patterns, (3) other mediating chemical factors and/or mechanism of action, and (4) the levels of the chemicals within and between plants.

METHODS AND MATERIALS

Eggs of *H. zea* were obtained weekly from Shell Development Company, Modesto, California. Eggs of *S. exigua* were obtained as needed from a laboratory colony. All diets used in this study were based on a semidefined artificial diet (No. 096L4) obtained from Bio-Serv Inc. (Frenchtown, New Jersey). Tomatine and cholesterol were purchased from Sigma Chemical Co. (St. Louis, Missouri).

For dose-response bioassays, tomatine was incorporated into artificial diets at different concentrations by dissolving the compound with the vitamins in the aqueous portion of the diet prior to preparing the complete diet. The concentrations used were 0.0, 0.1, 0.2, 0.3, and 0.4 $\mu\text{mol/g}$ diet wet weight for *S. exigua*, and 0.0, 0.5, 0.75, 1.0, and 1.5 $\mu\text{mol/g}$ for *H. zea*. In the tomato plant *L. esculentum* the average level of tomatine in the foliage is approximately 1.0 $\mu\text{mol/g}$ wet weight, with levels ranging from near 0.0 to >5 $\mu\text{mol/g}$ wet weight. The ability of cholesterol to modify tomatine toxicity was investigated by mixing equimolar ratios of tomatine and cholesterol into the diet based on the EC_{50} 's for tomatine (dietary concentration at which growth is reduced to 50% that of the controls) calculated from the above dose-response curves (EC_{50} 's used were 0.3 and 1.0 μmol tomatine/g diet wet weight for *S. exigua* and *H. zea*, respectively). Cholesterol was added to the dry portion of the diet prior to preparation, owing to its limited solubility in water.

Neonate larvae were sprinkled over 25-well plastic rearing trays (BioServ), with each well containing a small portion of diet. Glass plates were used as covers. Larvae were reared in an environmental chamber at 28°C with a 16:8 hr light-dark photoperiod and were thinned to one per well after three days. When larvae on control diet (2.4% casein) reached an average weight of 200–250 mg (mid-fifth instar; approx. 11 days) for *S. exigua* and 350–400 mg (mid-sixth instar; approx. 11 days) for *H. zea*, the bioassay was stopped and all of the larvae from each treatment were weighed. Throughout the experiments, feces were removed and new diet was added as needed. Each experiment was replicated three to five times ($N = 25/\text{replicate}$).

Diet-shift experiments were performed to determine the relative sensitivity of later-stage larvae of both species (third instar) to toxicosis from tomatine in the diet and to determine if toxicosis could be alleviated by transferring larvae to control diet. Neonate larvae ($N = 100$) were reared for five days on either control diet (C) or diet containing the appropriate EC_{50} of tomatine (T) for each species. After five days, half of each cohort was transferred to control diet and half transferred to tomatine-enriched diet. Larvae were then allowed to continue feeding for an additional four days. Thus, there were 50 larvae for each of four treatments, C/C, C/T, T/T, and T/C. All larvae were weighed daily from the third day on.

To determine the possible effects of tomatine on the efficiency of food utilization by *S. exigua* and *H. zea*, cohorts of larvae were first reared for five days on control diet (to a mean larval weight of approx. 15 mg for *S. exigua* and 20 mg for *H. zea*), then transferred to preweighed portions of diet containing tomatine or tomatine + cholesterol. The dietary concentrations of tomatine tested were 0.0, 0.15, 0.30, 0.60, 1.0, 3.0, and 0.0, 0.5, 1.0, 2.0, 3.0 $\mu\text{mol/g}$ for *S. exigua* and *H. zea*, respectively ($N = 45$ per treatment). A 1:1 tomatine-cholesterol mixture was used based on the EC_{50} for each species. After 36 hr, the larvae, frass, and remaining diet were weighed and nutritional indices (CI, AD, ECI, ECD) calculated as outlined by Waldbauer (1968) and Reese (1979). All calculations were based on wet weights, as the relative humidity in the rearing trays was near 100% and the water content of all test diets was identical.

Data were analyzed using one-way analysis of variance and linear regression with the Statistical Analysis System (SAS). Mean larval weights for different treatments and slopes of the regression lines calculated for the dose-response bioassays were separated using 95% confidence intervals according to Jones (1984). Species comparisons were made by converting larval weights to growth as a percentage of the controls.

RESULTS

Based on dose-response analysis of tomatine toxicity to both species (Figure 1), larvae of *S. exigua* were three times more sensitive to the chemical than were *H. zea* larvae. For *S. exigua* and *H. zea*, respectively, the EC_{50} 's were 0.3 ($y = -423x + 218$) and 1.0 ($y = -174x + 345$) $\mu\text{mol tomatine/g diet wet weight}$ (slopes' significant difference $P < 0.05$).

The effect of tomatine on larval growth was completely alleviated in the fruitworm *H. zea* by the addition of equimolar cholesterol into the diet. However, in *S. exigua* a significant amount of toxicity was maintained (Figure 1). A 1:1 tomatine-cholesterol level in the diet reduced larval weight gain similar to that of a tomatine concentration of 0.1 $\mu\text{mol/g diet alone}$, i.e., about 20% (significant difference from controls $P < 0.05$).

When larvae of *H. zea*, reared from neonate stage for five days on control diet, were transferred to diet with an EC_{50} concentration of tomatine, suppression of growth ensued almost immediately (Figure 2). After nine days, larvae that were continuously reared on control diet significantly outweighed those that had been transferred to diet containing tomatine (247 ± 10 mg vs. 194 ± 12 mg, $P < 0.05$). Larvae, reared for five days on diet containing tomatine, that were then transferred to control diet assumed rapid growth and weighed signif-

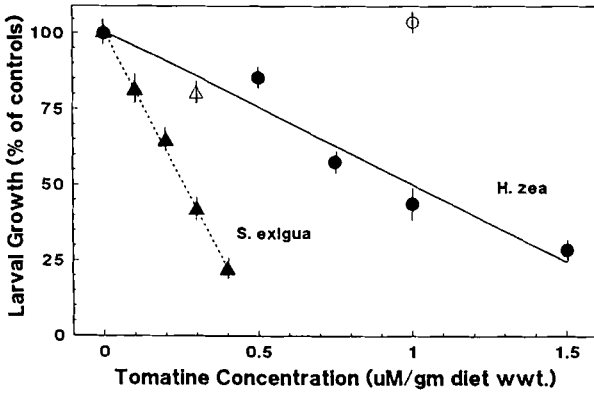


FIG. 1. Relationship between larval growth of *H. zea* (—●—) and *S. exigua* (---▲---), and concentration of tomatine added to artificial diets. Open symbols (○ and △) represent larval growth at the indicated tomatine concentration in combination with an equimolar level of cholesterol. Lines were fitted by linear regression; error bars = 95% confidence interval about each mean ($N = 75-100$).

icantly more after nine days than larvae that had remained on the tomatine diet (205 ± 9 mg vs. 140 ± 10 mg, $P < 0.05$).

This pattern of growth suppression and recovery did not hold for *S. exigua* (Figure 3). Transferring larvae after five days from control diet to diet with

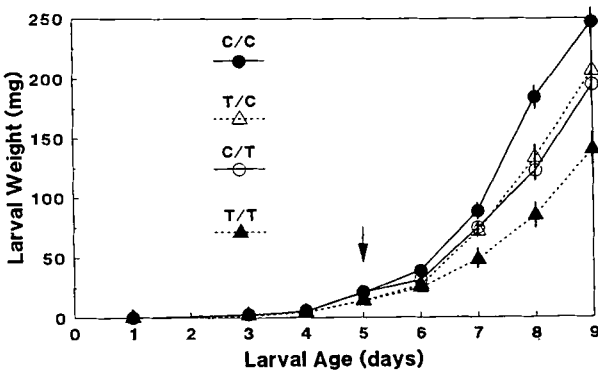


FIG. 2. Larval growth curves of *H. zea* reared on control diet (—●—) or diet with $1.0 \mu\text{mol}$ tomatine/g wet weight (=EC₅₀) (---▲---). Arrow indicates day on which half of the larvae being reared on control diet were transferred to diet with tomatine (○), and half of those on diet with tomatine were transferred to control diet (△). Error bars = 95% confidence interval about each mean ($N = 75-100$).

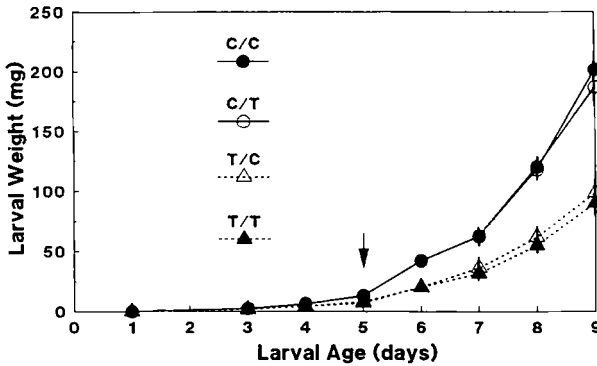


FIG. 3. Larval growth curves of *S. exigua* reared on control diet (—●—) or diet with $0.3 \mu\text{mol}$ tomatine/g wet weight ($=\text{EC}_{50}$) (---▲---). Arrow indicates day on which half of the larvae being reared on control diet were transferred to diet with tomatine (○), and half of those on diet with tomatine were transferred to control diet (△). Error bars = 95% confidence interval about each mean ($N = 75-100$).

tomatine, or vice versa, did not alter the larval growth curves. Larvae continued to grow as if the transfer had not occurred, i.e., similar to those reared exclusively on either control or tomatine diet, on whichever the larvae had started.

Results from experiments on efficiency of food utilization supported the results we obtained in the diet-shift experiment (Table 1). Third-instar larvae of *S. exigua* grew as well on diets containing up to two times the EC_{50} of tomatine as they did on control diet. Tomatine also did not affect feeding behavior of later-stage larvae of *S. exigua* (5 days old, third-instar), as evidenced by the fact that there were no significant differences in the consumption indices of larvae that fed on diets of different concentrations of tomatine, except a decrease at $3.0 \mu\text{mol/g}$ diet wet weight or 10 times the EC_{50} . Likewise, tomatine did not reduce approximate digestibility or the dietary utilization of the food for third-instar larvae, again except at the highest dose. Larvae reared on diet with cholesterol added at an equimolar level to tomatine (EC_{50}) also did not differ significantly from controls in weight gain or in any of the nutritional indices measured.

In contrast to *S. exigua*, third-instar larvae of *H. zea* that were shifted from control to tomatine diets showed a significant reduction in larval weight gain (Table 1). In all cases for *H. zea*, as the concentration of tomatine in the diet increased, larval weight gain decreased, going from a weight gain of $>40 \text{ mg}$ with $0 \mu\text{mol}$ of tomatine in the diet to approx. 25 mg at the EC_{50} , and $<18 \text{ mg}$ with $3.0 \mu\text{mol}$ tomatine/g diet. Tomatine also affected the efficiency of dietary utilization of the food. Both the ECI 's and the ECD 's decreased with increasing

TABLE I. RELATIONSHIPS BETWEEN DIETARY CONCENTRATIONS OF TOMATINE AND WEIGHT GAIN, DIETARY UTILIZATION OF FOOD, AND FOOD CONSUMPTION BY THIRD- TO FOURTH-INSTAR LARVAE OF *H. zea* AND *S. exigua*

Tomatine conc. ($\mu\text{mol/g}$ diet wet weight)	Weight gain (mg) \bar{X}^2	AD (%) \bar{X}	ECI (%) \bar{X}	ECD (%) \bar{X}	CI \bar{X}
<i>S. exigua</i> ($N = 45$)					
0.0	35.7a ^b	73.6a	21.6a	29.9a	3.3a
0.15	34.7a	74.4a	21.8a	30.2a	3.3a
EC ₅₀ 0.3	36.8a	71.4a	21.1a	30.5a	3.5a
0.3 + Chol. ^a	32.1ab	72.3a	17.6a	25.1a	3.7a
0.6	29.2ab	72.5a	18.5a	25.9a	3.3a
1.0	25.4b	74.3a	17.8a	25.4a	3.3a
3.0	12.8c	82.0b	13.3b	16.9b	2.7b
<i>H. zea</i> ($N = 45$)					
0.0	40.6a	76.1ab	15.7ab	20.9b	4.7a
0.5	32.6b	77.2ab	14.3b	18.8b	4.7a
EC ₅₀ 1.0	24.8c	77.7ab	12.4c	16.2c	4.8a
1.0 + Chol.	44.6a	74.0b	17.5a	24.3a	4.3a
2.0	19.3d	79.3a	9.3d	11.9d	5.6b
3.0	17.7d	80.1a	8.7d	11.2d	5.5b

^aCholesterol was added to these diets at equimolar levels to that of the tomatine.

^bMeans followed by the same letter are not significantly different as determined by an overlap of the 95% confidence limits.

tomatine concentrations. Assimilation of the food (AD) tended to increase with increasing amounts of tomatine in the diet. The inhibition of growth was not the result of inhibition of feeding behavior because there were no significant differences in the consumption indices at levels up to the EC₅₀. At two and three times the EC₅₀, the CI actually increased, potentially in compensation for the lowered efficiency of food utilization. The addition of cholesterol into the diet completely alleviated tomatine toxicity. Larvae of *H. zea* reared on control diet or diet with a 1:1 tomatine-cholesterol mixture did not differ significantly in weight gain, CIs, ADs, or ECIs.

DISCUSSION

Glycoalkaloids have been implicated as resistance factors in the solanaceous crops potato and tomato against a variety of insects based on the ability of these chemicals to alter the insects' behavior and/or physiology (Kuhn and Low, 1955; Sturckow and Low, 1961; Dahlman and Hibbs, 1967; Raman et al., 1979; Sinden et al., 1980; Elliger et al., 1981). Tomatine, the major gly-

coalkaloid in tomato plants (Schreiber, 1968), has been discussed as a potential chemical basis of resistance in tomatoes against the Colorado potato beetle *L. decemlineata* (Sinden et al., 1978), the tomato fruitworm *H. zea* (Juvik and Stevens, 1982), and the Mediterranean fruit fly *Ceratitidis capitata* (Wiedemann) (Chan and Tam, 1985).

Our concern has been to assess more thoroughly the potential of tomatine to serve as a basis of resistance against *S. exigua* and *H. zea*, the two primary pests of commercial tomatoes in California (Lange and Bronson, 1981). Juvik et al. (1982) surveyed the genus *Lycopersicon* and found significant genetic variation in foliar tomatine content among 27 accessions tested. Based on these findings, Juvik and Stevens (1982) chose three accessions with different foliar tomatine levels to test the relationship between fruit tomatine content and resistance to larval *H. zea* and *S. exigua*. They found a highly significant, negative correlation between the tomatine content of the fruit and a reduction in larval growth of *H. zea*, but stated that larvae of *S. exigua* were unaffected by the level of tomatine in their diet. These results are inconsistent with our findings that larvae of *S. exigua* were three times more sensitive to dietary tomatine than were larvae of *H. zea* (Figure 1).

Two factors that may explain this apparent discrepancy are (1) the level of phytosterols in tomato foliage and fruit, and (2) larval age and/or prior exposure to tomatine. First, we and other researchers have demonstrated that saponin toxicity can be attenuated or even alleviated by addition of equimolar or supra-molar levels of certain phytosterols to an insect's diet. Therefore, one must examine the composite level of both chemicals before an accurate assessment of toxicity can be given (Shany et al., 1970b; Campbell and Duffey, 1979, 1981; Kelley et al., 1988). Unfortunately, Juvik and Stevens (1982) did not measure the level of sterols in either foliage or fruit. Second, data from our experiments on diet transfers and nutritional indices showed later-stage larvae of *S. exigua* to be insensitive to dietary tomatine (Figure 3; Table 1), whereas larvae of *H. zea* were sensitive at all instars tested (first to third) (Figure 2; Table 1). Thus, by the time larvae initially fed on foliage with a low tomatine content and/or tomatine-to-sterol ratio matured enough to move from feeding on foliage to feeding on fruit (as in the experimental procedure of Juvik and Stevens, 1982), *S. exigua* would no longer be sensitive to tomatine. Hence, one would expect to find only a correlation between fruit tomatine content and reduced larval growth for *H. zea*.

The levels of tomatine (Juvik et al., 1982) and sterols (Campbell and Duffey, 1981) in tomato plants have been shown to vary significantly not only between varieties but between and within plants of a single variety (Duffey and Bloem, 1986; Duffey, unpublished data). Also, larvae of *H. zea* and *S. exigua* are quite mobile and may change their location on a plant to move to new leaves

between successive feeding bouts, to find more favorable microhabitats, and/or to feed on green fruit (tomatoes: Brendler et al., 1985; L.T. Wilson, personal communication; cotton: Baldwin, 1972; Wilson et al., 1980). This suggests that if one is going to inhibit effectively the growth of one or both species throughout larval development, the tomatine/sterol levels in all aerial portions of the plant should be similar (perhaps between an EC_{50} for *H. zea* and *S. exigua*) and/or at least higher in green fruit so that larvae neither develop insensitivity to tomatine nor recover by moving to plant parts containing less tomatine.

The differential sensitivity of herbivorous species to plant natural products must also be considered when assessing the impact of such chemicals on natural enemies (Duffey and Bloem, 1986; Duffey et al., 1986). The ichneumonid wasp *H. exiguae* is a primary biological control agent of both *H. zea* and *S. exigua* larvae in tomatoes in California (Oatman et al., 1983). From the work of Campbell and Duffey (1979, 1981) and additional work by Duffey and Bloem (1986), the sensitivity of the wasp and noctuids to tomatine, in decreasing order, is *S. exigua* > *H. exiguae* > *H. zea*. Thus, the use in planta of an appropriate ratio of tomatine to sterols (e.g., EC_{50} or EC_{75}) directed against *S. exigua* would likely be compatible with biological control efforts utilizing this wasp. However, our dose-response bioassays show that such levels would have little or no effect on *H. zea*. Levels of tomatine effective against *H. zea*, on the other hand, may be incompatible with the use of this wasp (Campbell and Duffey, 1979, 1981; Duffey and Bloem, 1986). Any incompatibility may be compounded if pest populations evolve tolerance to these antibiotics while parasitoid populations remain sensitive (Flanders, 1942; Mueller, 1983; Duffey and Bloem, 1986).

The above considerations underline the need to better understand at a physiological level the effect of plant allelochemicals on an organism(s) before one tries to implicate or utilize specific plant chemicals as bases of defense or resistance. As noted by Birk and Peri (1980), the mode of action of saponins in relation to insect growth and survival is complex and poorly understood. Although several modes of action have been proposed (Shany et al., 1974; Birk and Peri, 1980; Campbell and Duffey, 1981), it is becoming increasingly clear that at least some of their biological activity in certain insects is coupled to their ability to complex with phytosterols, such as cholesterol (Shany et al., 1970b; Campbell and Duffey, 1981; Kelley et al., 1988). In our experiments, the addition of equimolar levels of cholesterol to tomatine-enriched diets completely alleviated (*H. zea*) or significantly reduced (*S. exigua*) tomatine toxicity (Figure 1). The differences observed in our nutrition and feeding studies, coupled with significantly different slopes of the dose-response curves, suggest that it may be simplistic to argue for a single mode of action for tomatine in these species. Additional research is needed, however, to determine whether sterol binding is

directly related to the mode of action of tomatine, or whether binding simply limits the expression of tomatine toxicity.

Resistance of plants to insects is usually thought of as the action of a discrete, definable state or entity of the plant upon a given pest species. We think, as do others (e.g., Price et al., 1980; Futuyama, 1983; Price, 1983; Whitham, 1983; Kogan, 1986), that current theories on plant defensive strategies fail to address adequately the interaction among coingested plant products, the existence of multiple pest species, and the effects of plant defenses as extended through multitrophic systems. The concerns and difficulties we raise for tomatine are not unique. Two phenolics, rutin and chlorogenic acid, have also been proposed as a basis of resistance in tomatoes (Isman and Duffey, 1982). These chemicals are differentially toxic to *H. zea* and *S. exigua*, are modified in toxicity by both polyphenol oxidase activity and dietary protein, and vary significantly in amount within and among plants (Duffey and Bloem, 1986). Likewise, tomato proteinase inhibitors are differentially toxic to *H. zea* and *S. exigua*, and their toxicity depends on the quantity and quality of dietary protein (Broadway and Duffey, 1986a,b, 1988). Clearly, the ability to utilize plant natural products predictively in host-plant resistance programs will require a more profound integration of plant phenology, insect feeding behavior, and the nutritional physiology and toxicology of the chemicals involved.

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AGGREGATION PHEROMONE COMPONENTS IN
Drosophila mulleri
A Chiral Ester and an Unsaturated Ketone

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Abstract—Existence of an aggregation pheromone was demonstrated in *Drosophila mulleri*. Mature males produce at least two compounds that are lacking from females and newly emerged males and that attract both males and females in a wind-tunnel bioassay. These compounds are (*S*)-(+)-2-tridecanol acetate and (*Z*)-10-heptadecen-2-one. Both were synthesized, and the flies responded to the synthetic compounds as well as to fly-derived preparations. The flies also responded to racemic 2-tridecanol acetate but not to the pure *R* enantiomer. A more polar, very volatile attractant was also extracted from both sexes of *D. mulleri* but was not identified.

Key Words—*Drosophila mulleri*, Diptera, Drosophilidae, aggregation, pheromone, chirality, enantiomers, ester, ketone, (*S*)-(+)-2-tridecanol acetate, (*Z*)-10-heptadecen-2-one.

INTRODUCTION

Aggregation pheromones have been demonstrated in a number of species of the genus *Drosophila* (Diptera: Drosophilidae) (Moats et al., 1987; Schaner et al., 1987; Bartelt et al., 1986, 1987; and references therein). In these species, sexually mature males possess compounds that attract both males and females in a laboratory wind-tunnel bioassay. These compounds are lacking in females and in newly emerged males. The pheromone components include hydrocarbons,

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esters, and ketones, and mixtures of compounds are often required for optimal responses. Volatiles from fermenting food media usually synergize the pheromones.

Research on pheromones of *Drosophila* species was continued with *D. mulleri* Patterson. A comparison between *D. mulleri* and *D. hydei* (see Moats et al., 1987) was of special interest because both belong to the same (*repleta*) species group. *D. mulleri* was found to possess pheromone components not previously encountered in other *Drosophila*.

METHODS AND MATERIALS

Flies, Extracts, and Bioassays. The *D. mulleri* culture was obtained from the National *Drosophila* Resource Center, in Bowling Green, Ohio (stock number 15081-1371). The flies were reared on Formula 4-24 Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, North Carolina).

The age of maturity of male and female flies was determined as described by Moats et al. (1987), so that appropriate ages for extraction and bioassay could be chosen. We found that males of *D. mulleri* become mature at 5-6 days and females at 2-3 days. In other *Drosophila* species we have studied, pheromone production begins in males as sexual maturity is approached, but flies of any age have usually responded to the pheromones. Thus, to obtain pheromone, flies were not extracted until 6-7 days of age, but 1- to 4-day-old flies were used for most bioassays because the immature males would be less likely to possess pheromones that could compete with bioassay treatments. Flies to be extracted were segregated by sex within one day of emergence and extracted by soaking them overnight in hexane at room temperature.

Bioassays were performed in a wind-tunnel olfactometer essentially as described earlier (Bartelt and Jackson, 1984). About 16 hr before bioassay tests began, ca. 1000 flies were added to the olfactometer. As with the previously studied species, a starvation period was necessary before flies would respond to pheromones. Each bioassay treatment was applied to a filter paper strip lining the lip of a glass vial. A drop of water was added to the bottom of each bioassay vial to arrest responding flies (Bartelt and Jackson, 1984). Two such vials, with treatments to be compared, were placed in the upwind end of the olfactometer. After 3 min the vials were capped, and the captured flies were counted and, in selected experiments, sexed. Tests were performed throughout the day at ca. 10-min intervals. In experiments with more than two treatments, they were tested in all possible pairs (a balanced incomplete block design). Statistical analysis followed the method of Yates (1940) and was done on data transformed as $\log(X + 1)$ to stabilize variance.

Chromatography and Spectra. Crude hexane extracts were chromato-

graphed initially on open columns of silicic acid and eluted with two void volumes of each of the following solvents: hexane; 5%, 10%, and 50% ether in hexane; and 10% methanol in methylene chloride.

All gas chromatography (GC) was conducted on a Varian 3700 using He as the carrier gas. For preparative GC, a 1.3-m \times 4-mm 5% SE-52 column was used, and the effluent was monitored with a thermal conductivity detector. Three columns were utilized for capillary GC: a 30-m \times 0.25-mm DB-1, a 15-m \times 0.32-mm DB-5, and a 30-m \times 0.25-mm DB-225 (J & W Scientific, Rancho Cordova, California). Peaks were monitored with a flame ionization detector. Various temperature programs were used, as described below.

High-performance liquid chromatography (HPLC) was conducted isocratically with a Waters 6000A pump and a Waters 401 differential refractometer. Columns included a 25-cm \times 4.6-mm ID Adsorbosphere silicic acid column (Applied Science, Deerfield, Illinois), eluted with 5% ether in hexane, and a 25-cm \times 4.6-mm ID silicic acid column, coated with AgNO₃ as described by Heath and Sonnet (1980), and eluted with toluene.

Electron impact mass spectra were obtained on a VG-MM16F mass spectrometer. Samples were introduced through a capillary GC column.

Synthetic Esters. (\pm)-2-Tridecanol acetate was prepared by treating dodecanal with methylmagnesium bromide in ether, followed by acetylation of the resulting alcohol with acetic anhydride in pyridine. After chromatography on an open column of silicic acid and preparative GC, the ester was >98% pure by capillary GC. The ester was diluted with hexane to 10 ng/ μ l for bioassays. A portion of the (\pm)-2-tridecanol was set aside for chromatographic studies.

(*S*)-2-Tridecanol was synthesized (Figure 1) from a commercially available, optically active starting material, ethyl (*S*)-lactate (I) (Sigma Chemical Co., St. Louis, Missouri). Synthetic steps were performed as described in the literature for analogous reactions: After conversion to a tetrahydropyranyl (THP) ether (II) (Miyashita et al., 1977), the ester function was reduced with lithium aluminum hydride (Mori, 1976), and the resulting primary alcohol (III) was esterified with *p*-toluenesulfonyl chloride to form the tosylate (IV) (Mori, 1976). Decylmagnesium bromide was linked to this tosylate in the presence of dilithium tetrachlorocuprate to form the THP ether of (*S*)-2-tridecanol (V) (Suguro and Mori, 1979). Finally, the tetrahydropyranyl protecting group was removed (Miyashita et al., 1977). The crude (*S*)-2-tridecanol (VI) crystallized at ca. -10°C from a 20% solution in pentane. By GC, the purity of the recovered product was 88%.

All reactions were monitored by GC and mass spectrometry. All were essentially quantitative except for the alkylation of the tosylate, for which the yield was ca. 50%. Intermediate products were not purified before subsequent reactions, except to dry (Na₂SO₄) and to remove solvents.

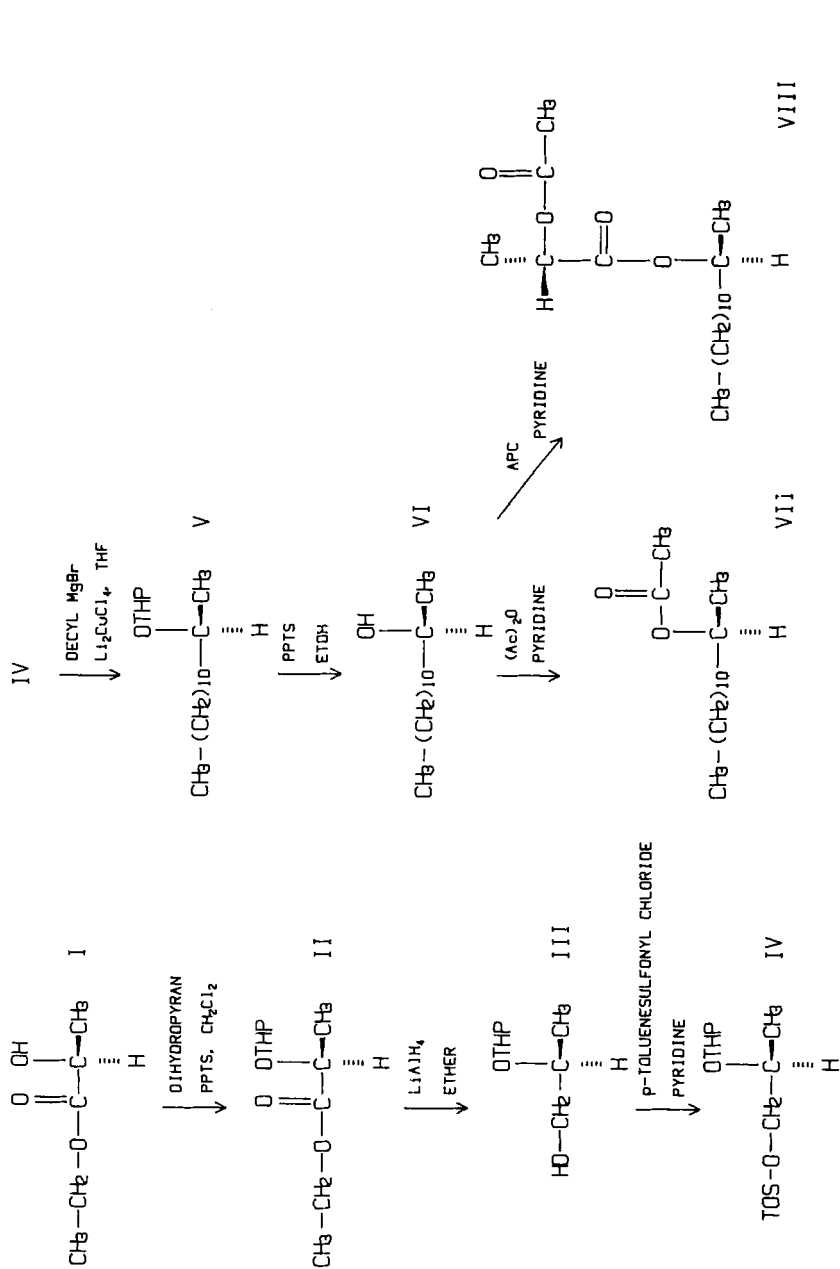


FIG. 1. Synthetic scheme for (S)-(+)-2-tridecanol, its acetate, and its (S)-2-(acetyloxy)propanoate. Abbreviations: THP = tetrahydropyranyl group, PPTS = pyridinium *p*-toluenesulfonate, TOS = *p*-toluenesulfonyl group, APC = (S)-2-(acetyloxy)propanoyl chloride.

(*R*)-2-Tridecanol was prepared in an identical manner except that the starting material was methyl (*R*)-lactate (Sigma).

(*S*)-2-Tridecanol was either converted to an acetate for bioassays or was derivatized as described below for GC studies of optical purity and stereochemical configuration. The acetate (Figure 1, VII) was formed by treatment with acetic anhydride in pyridine. After purification by chromatography on silicic acid and preparative GC, the ester's purity was >99%, by capillary GC. Approximately 10 mg of pure ester were prepared. The ester had an identical mass spectrum and GC retention (DB-1, DB-5) as the racemic synthetic (\pm)-2-tridecanol acetate. The specific rotation was determined: $[\alpha]_D^{23} = +4.6^\circ$ ($c = 0.57\%$, hexane) (literature value: $[\alpha]_D^{20} = +4.63^\circ$, absolute configuration not given, Pickard and Kenyon, 1914).

(*R*)-(-)-2-Tridecanol acetate was likewise prepared from the (*R*)-2-tridecanol. The measured purity of the ester was >99% and the optical rotation, $[\alpha]_D^{23} = -4.9^\circ$ ($c = 0.57\%$, hexane). GC retention and mass spectrum were as for the *S* isomer. Both enantiomeric esters were diluted with hexane for bioassays.

Studies of Optical Purity and Absolute Configuration. Diastereomeric esters (Figure 1, VIII) were formed from the synthetic 2-tridecanols so that optical purity could be analyzed on an achiral GC column. The (*S*)-(2-acetyloxy)propanoates (acetyl lactates) were prepared essentially as described by Slessor et al. (1985). The derivatizing reagent was (*S*)-(2-acetyloxy)propanoyl chloride (APC). GC analysis was on the DB-5 capillary column, temperature programmed from 120°C to 150°C at 5°C/min.

To derivatize the 2-tridecanol acetate from *D. mulleri*, the ester was first reduced with LiAlH_4 . To ca. 10 μg of purified *D. mulleri* ester in 20 μl ether was added 15 μl LiAlH_4 reagent (1.0 M in ether). After 10 min at room temperature, the vial was cooled over ice and 2 drops of H_2O were added to decompose excess reagent. The organic layer was transferred to a clean vial and stripped to dryness under N_2 ; then the alcohol was derivatized with APC.

Synthesis of Ketones. (*Z*)-9-Hexadecenoic acid (50 mg, 0.2 mmol, Sigma) in 2 ml dry ether was cooled to 0°C. Methylolithium in ether (0.8 mmol, 0.6 ml) was added in one portion. The mixture was warmed to room temperature, refluxed for 20 min, then cooled to 0°C, after which 1 ml water was added very slowly. The ether layer was washed with dilute HCl, water, and NaHCO_3 solution, then dried over Na_2SO_4 . The resulting (*Z*)-10-heptadecen-2-one was purified on an open column of silicic acid and diluted with hexane for bioassay. The purity was 99+ % by capillary GC. 2-Heptadecanone was similarly prepared in 99+ % purity from palmitic (hexadecanoic) acid.

Preliminary Experiments with Volatile, Polar Attractants. Apparatus was assembled to allow evaporation of a sample (up to 500 μl) under a gentle stream of nitrogen, so that the vapors passed through a $2.5 \times 25\text{-mm}$ column of 20/

35 mesh Tenax porous polymer (Applied Science). Both the fly-derived 10% MeOH-CH₂Cl₂ fraction and a CH₂Cl₂ rinse of rearing medium (inoculated with yeast but without *Drosophila*) were treated in this way. Each sample was reconstituted with an equal volume of fresh solvent immediately as the sample went to dryness, and the Tenax column was eluted with an equivalent volume of pentane. The reconstituted samples and Tenax column rinses were compared to the original samples by bioassay. The Tenax column rinses were examined by GC and mass spectrometry.

RESULTS AND DISCUSSION

Bioassay Characteristics. When the flies were first put into the olfactometer, they formed tight aggregations low on the sides and moved about very little. After starvation for 14–16 hr at a temperature of 24–25°C, these groups dispersed and the flies became more active, usually with ca. 20–30 flies in flight at any given time. Response to an active treatment was usually by an upwind, zigzag, hovering flight, followed by alighting on the vial.

Bioassays of Crude Extracts and Fractions. The crude hexane extract of mature male *D. mulleri* was active in the bioassay (Table 1A), and three fractions derived from this extract were significantly active. Based on standards, the first (eluted with 5% ether-hexane) had the polarity of esters while the second (eluted with 10% ether-hexane) had the polarity of ketones. The last was considerably more polar (eluted with 10% methanol-methylene chloride). Surprisingly, the female-derived extract was also very active (Table 1B), and this activity was located in the most polar fraction. Thus *D. mulleri* was like the previously studied species in that mature males had attractive nonpolar compounds that were lacking in mature females, but *D. mulleri* was unlike the other species in that very active, relatively polar compounds(s) were present in both sexes.

Further purification of the male-derived 5% and 10% ether-hexane fractions was accomplished by preparative GC. The activity from the 5% ether-hexane fraction was retained slightly longer than an alkane of 16 carbons on the nonpolar preparative GC column, and the activity from the 10% ether-hexane fraction was similar in retention to an alkane of 19 carbons. No other GC fractions had significant bioassay activity. Subsequent HPLC on silicic acid produced only one active region from each GC fraction. The activity originally from the 5% ether-hexane silicic acid fraction eluted 5.5–6.5 ml after injection, while that from the 10% ether-hexane fraction eluted at 10–11 ml. Based on standards, these retentions supported that the activity in the former fraction was due to an ester, and the latter, a ketone.

Ester Component. After preparative GC and HPLC, the active ester com-

TABLE 1. ACTIVITY OF SILICIC ACID FRACTIONS RELATIVE TO CONTROLS AND TO MALE-DERIVED CRUDE EXTRACT

Treatment ^a	Mean bioassay catch ($N \geq 8$) ^b		
	Treatment	Control	Male-derived crude extract
A. Male-derived fractions			
Hexane	8.5	8.7	57.5***
5% Ether-hexane	13.6***	3.8	23.4***
10% Ether-hexane	8.0***	2.8	33.4***
50% Ether-hexane	5.5	6.0	49.1***
10% Methanol-methylene chloride	23.0***	1.0	23.8***
B. Female-derived extract and fractions			
Crude hexane extract	29.1***	5.0	21.7***
Hexane fraction	4.4	4.0	52.2***
5% Ether-hexane	3.2	4.2	40.9***
10% Ether-hexane	6.3	4.4	42.6***
50% Ether-hexane	2.3	4.3	22.9***
10% Methanol-methylene chloride	27.5***	2.9	22.0***

^aOne fly equivalent per test. Each row is a balanced incomplete block experiment, in which the test treatment, the control, and the male-derived crude extract are tested in pairs, in all possible combinations.

^bIn each row, means followed by *** are significantly different from the control at the 0.001 level (*t* tests). No other differences were significant at even the 0.05 level.

ponent was >98% pure, by capillary GC. Mature males possessed ca. 300 ng of this compound, but it was lacking in females and newly emerged males. In the mass spectrum, no molecular ion was seen, but fragments at m/z 87 (40%) and 182 (15%, M-60) suggested an acetate of the secondary alcohol, 2-tridecanol. The base peak was m/z 43, and peaks also occurred at 198 and 199 (both ca. 1%, M-44 and M-43, respectively). Racemic 2-tridecanol acetate and the *D. mulleri* compound had identical GC retention times (DB-1) and mass spectra. Both reacted with methanolic KOH, resulting in a GC peak with the same retention as authentic 2-tridecanol. Racemic 2-tridecanol acetate was active in the bioassay (Table 2A).

The fly-derived 2-tridecanol acetate, having one asymmetric center, was analyzed for optical purity and absolute configuration (summarized in Figure 2). The two diastereomers derived from racemic 2-tridecanol were easily separated on the DB-5 capillary column. Based on the synthetic standards, the first of these peaks (Kovats index = 1966) corresponded to the *R* configuration, while the peak at KI = 1982 represented the *S* configuration. The derivative

TABLE 2. KEY BIOASSAY RESULTS RELATING TO IDENTITIES OF NONPOLAR PHEROMONE COMPONENTS

Treatment	Mean bioassay catch ^a
A. Preliminary identification of ester component (<i>N</i> = 74).	
(±)-2-Tridecanol acetate (50 ng)	26.5a
Control	4.9b
B. Determination of active enantiomer (<i>N</i> = 40).	
(<i>S</i>)-(+)-2-Tridecanol acetate (25 ng)	22.9a
(<i>R</i>)-(-)-2-Tridecanol acetate (25 ng)	5.6b
Control	6.3b
C. Comparison of male-derived "ester" fraction and synthetic ester (<i>N</i> = 12). (Both treatments had 300 ng 2-tridecanol acetate per test).	
(<i>S</i>)-(+)-2-Tridecanol acetate	14.2a
Male-derived 5% ether-hexane fraction	14.2a
Control	3.1b
D. Comparison of male-derived "ketone" fraction and synthetic ketone (<i>N</i> = 16). [Both treatments had 0.7 ng (<i>Z</i>)-10-heptadecen-2-one per test.]	
(<i>Z</i>)-10-Heptadecen-2-one	14.1a
10% Ether-hexane fraction	19.9a
Control	2.9b
E. Activity of two 17-carbon ketones, alone and in combination (<i>N</i> = 12). (Each ketone used at 8 ng per test)	
(<i>Z</i>)-10-Heptadecen-2-one	14.9b
2-Heptadecanone	4.9c
(<i>Z</i>)-10-Heptadecen-2-one + 2-heptadecanone	25.4a
Control	4.4c

^aIn each experiment, means followed by the same letter not significantly different at the 0.05 level (LSD method).

prepared from the *D. mulleri* pheromone had a Kovats index of 1982 and a high degree of optical purity (enantiomeric excess calculated from peak integrations was 98.7%). By comparison to the synthetic standards, the *D. mulleri* pheromone has the *S* configuration. The enantiomeric excess represents a minimum, as the departure from 100% could be due to optical impurity of the derivatizing reagent and/or slight racemization occurring during chemical procedures.

D. mulleri responded in the bioassay only to the enantiomer of 2-tridecanol acetate which they produce (Table 2B). The *S* isomer was clearly active, while the *R* isomer was not. That the *R* isomer was not repellent was consistent with

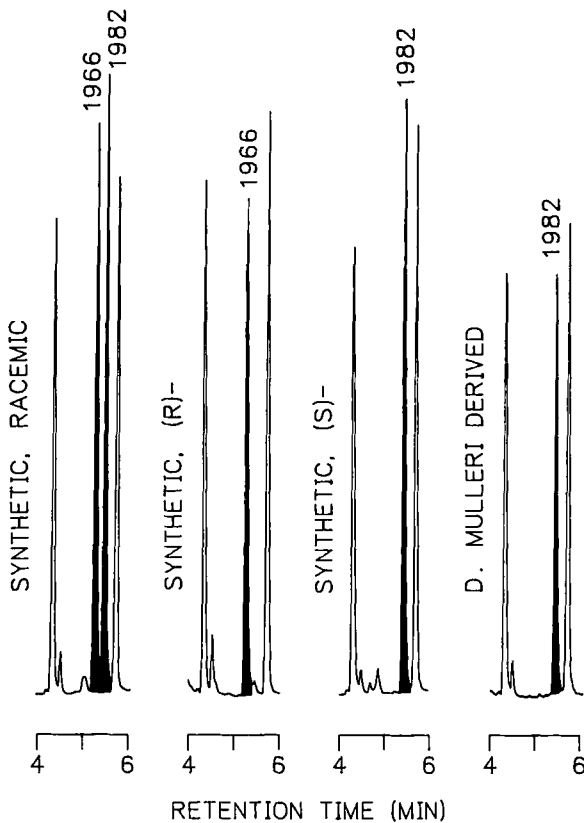


FIG. 2. GC analysis of optical purity and absolute configuration of the 2-tridecanol acetate produced by *D. mulleri*. Shaded peaks represent (*S*)-2-(acetyloxy)propanoate derivatives of synthetic and fly-derived 2-tridecanol. Kovats indices are indicated at peak apices. The peaks at ca. 4.3 and 5.8 min are *n*-alkanes of 19 and 20 carbons, respectively, conjoined as retention standards. The DB-5 capillary column was 120°C initially and was increased at 5°C/min.

the activity of the racemic synthetic ester. (*S*)-2-Tridecanol acetate adequately accounts for the activity of the male-derived 5% ether-hexane fraction (Table 2C). In this experiment both treatments had equivalent amounts of the ester.

Chiral pheromones have been demonstrated in a number of insect groups. One common pattern is that insects respond only to the optical isomer that they produce but that the other enantiomer is not repellent (Mori, 1985), a pattern which has now been demonstrated in the *Drosophila* also.

Ketone Component. The active "ketone" fraction, after preparative GC

and HPLC on silicic acid, contained two peaks, by capillary GC. Both were small peaks, representing ca. 1 ng/mature male. The latter of the two peaks was identical in GC retention and mass spectrum with 2-heptadecanone [key spectral peaks were at m/z 43 (100%), 58 (80%), and 254 (4%)]. The other peak eluted slightly earlier on the nonpolar DB-1 capillary column, and its mass spectrum indicated a molecular weight of 252 (2%), 2 mass units less than 2-heptadecanone. Fragments still appeared at m/z 43 (100%) and 58 (26%); thus an unsaturated methyl ketone seemed likely. (*Z*)-10-Heptadecen-2-one was synthesized as a standard for comparative purposes. This ketone was accessible in one step from the readily available (*Z*)-9-hexadecenoic (palmitoleic) acid. Fortuitously, this ketone had the same retention on three GC columns as the *D. mulleri* ketone (KI = 1860, 1873, and 2243 on DB-1, DB-5, and DB-225, respectively), and the mass spectra and retentions on the AgNO₃ HPLC column were identical also. Since these chromatographic methods are sensitive to double-bond position and configuration, the data strongly supported (*Z*)-10-heptadecen-2-one as the structure of the unknown compound. Because of the minute amount of ketone in each male, confirmation of double bond location by ozonolysis was not attempted.

(*Z*)-10-Heptadecen-2-one was active in bioassay and accounts fairly well for the activity of the male-derived 10% ether-hexane fraction (Table 2D). 2-Heptadecanone, also found in *D. mulleri* males, was not active in the bioassay by itself, although a mixture of 2-heptadecanone + (*Z*)-10-heptadecen-2-one was somewhat more active than the unsaturated ketone alone (Table 2E).

The extracts were subsequently examined for 2-tridecanone and 2-pentadecanone, pheromone components in the related species, *D. hydei* (Moats et al., 1987). Traces of both ketones were found (2–4 ng/mature male), but the synthetic ketones were not active in the bioassay. None of the ketones were detected in extracts of female flies.

Synergistic Activity of (S)-(+)-2-Tridecanol Acetate and (Z)-10-Heptadecen-2-one. Dose-response data for various mixtures of (*S*)-2-tridecanol acetate and (*Z*)-10-heptadecen-2-one are given in Table 3. The response generally increased with increases in either component, although there was little difference between the 300- and 3000-ng levels of the ester. On a weight-for-weight basis, the ketone was the more active compound. For example, 10 ng of ketone elicited a stronger response than even 3000 ng of ester.

Response by Sex. Both sexes responded readily to all preparations tested. For example, in a paired comparison experiment with the crude hexane extract of mature males (1 equivalent), (*S*)-2-tridecanol acetate (300 ng), (*Z*)-10-heptadecen-2-one (10 ng), and controls, the mean captures (and percent females) for the treatments were 38.7 (45%), 11.8 (47%), 29.5 (45%), and 2.0 (67%), respectively ($N = 6$).

Polar Attractant. Despite concerted efforts, the polar attractant(s) in the

TABLE 3. RELATIVE ACTIVITIES FOR VARIOUS MIXTURES OF (S)-(+)-2-TRIDECANOL ACETATE AND (Z)-10-HEPTADECEN-2-ONE^a

Amount of (Z)-10-heptadecen-2-one (ng)	Amount of (S)-(+)-2-tridecanol acetate (ng)				
	0	3	30	300	3000
0.0	0 ^b	8	19	50	57
0.1	3 ^c	28	35	74	58
1	14	39	58	100 ^d	140
10	92	151	170	226	294
100	258	364	387	504	555

^aEach test mixture compared to solvent controls and the standard mixture (300 ng (S)-(+)-2-tridecanol acetate plus 1 ng (Z)-10-heptadecen-2-one) in a balanced incomplete block experiment ($N = 8$). Relative activity (RA) based on means from these experiments:

$$RA = 100 \times (\text{test mixture} - \text{control}) / (\text{standard mixture} - \text{control}).$$

^bBy definition, RA for control = 0. Overall, the mean bioassay catch for controls was 4.5 flies per 3-min test.

^cAll samples except this one attracted significantly more flies than the control ($P < 0.01$).

^dBy definition, RA for standard mixture (ca. 1 male equivalent) was 100. Overall, the mean bioassay catch for this standard was 34.8 flies per 3-min test.

10% MeOH-CH₂Cl₂ fraction was (were) not identified. The activity was extremely volatile, compared to the ester and ketone pheromone components. Evaporating the polar fraction carefully to dryness under N₂ and immediately reconstituting the fraction with fresh solvent resulted in the loss of ca. 80% of the bioassay activity (Table 4). When the vapors from the evaporating fraction were directed through a column of Tenax, the activity could be recovered by rinsing the column with pentane. (Evaporation of control solvent through the Tenax trap did not produce a trap rinse that was active in bioassay). Examination of the active trap rinse by GC revealed many minute peaks, but there was little consistency among runs and there was not enough material for definitive mass spectral analysis. It is likely that a mixture of compounds is involved in the response.

The polar attractant was unlike any of the previously studied *Drosophila* pheromone fractions in that those from both sexes were highly active, instead of only those from males. It is possible that this activity is related to the diet medium rather than being pheromonal, since the flies could pick up compounds from their food. A CH₂Cl₂ rinse of diet medium, which was never exposed to *Drosophila*, was similarly attractive in bioassay, and when it was evaporated through Tenax, the rinse from the Tenax column was also active (Table 4). The involvement of the volatile attractants in the ecology of this fly species will require additional research.

TABLE 4. VOLATILE ATTRACTANT IN FLY-DERIVED POLAR FRACTION AND EXTRACT OF REARING MEDIUM

Treatments compared	Mean bioassay catch ^a	
	<i>D. mulleri</i> polar fraction ^b	CH ₂ Cl ₂ extract of rearing medium
A. Loss of activity upon evaporation of solvent from sample ^d		
Original sample	35.1a (N = 16)	22.1a (N = 4)
Evaporated, reconstituted	7.3b	5.0b
Solvent control	1.6c	1.0c
B. Transfer of activity to Tenax as sample evaporated ^e		
Original sample	25.2a (N = 8)	21.4a (N = 8)
Rinse of Tenax trap	26.1a	13.9a
Solvent control	3.4b	0.9b

^aEach group of three means represents a balanced incomplete block experiment. Values followed by the same letter not significantly different (LSD, 0.05).

^bResults for fractions from males and females nearly identical; combined data presented.

^cRearing medium inoculated with yeast, but no *Drosophila* present.

^dSamples evaporated gently under stream of N₂; samples reconstituted with fresh solvent immediately after going to dryness.

^eVolatiles from evaporating sample passed through Tenax trap; collection terminated when sample went to dryness. Tenax trap rinsed with pentane.

D. mulleri is like the other, previously studied *Drosophila* species in that attractants exist that are found only in the mature males. *D. mulleri* is similar to *D. hydei* (also of the *repleta* group) in that both species have ester and ketone pheromone components. In *D. hydei*, however, the ester is ethyl tiglate (Moats et al., 1987), while *D. mulleri* uses (*S*)-(+)-2-tridecanol acetate. Indeed, the ester of *D. mulleri* is probably biosynthetically related to the ketone component of *D. hydei*, 2-tridecanone. Yet *D. mulleri* still retains a methyl ketone component, although it is four carbons larger than that from *D. hydei* and is unsaturated.

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ISOLATION, IDENTIFICATION, AND BIOSYNTHESIS OF COMPOUNDS PRODUCED BY MALE HAIRPENCIL GLANDS OF *Heliothis virescens* (F.) (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Extracts of the intact hairpencil glands and hairs from the hairpencil glands of males of *Heliothis virescens* (F.) were analyzed by capillary gas chromatography (GC) and by GC-mass spectroscopy. These analyses indicated that hexadecanyl acetate (212.4 ng/male), hexadecanol (22.3 ng/male), (*Z*)-11-hexadecenyl acetate (3.5 ng/male), octadecanyl acetate (14.2 ng/male) octadecanol (7.5 ng/male), tetradecanoic acid (2.7 ng/male), hexadecanoic acid (22.3 ng/male), and octadecanoic acid (6.5 ng/male) were present in the extracts. These compounds also were found in volatiles released from the hairpencil glands. In addition, GC analysis using both polar and apolar capillary columns indicated that extracts of the glands and extracts of the hairs from the hairpencil glands contained small amounts of tetradecanyl acetate, (*Z*)-9-tetradecenyl acetate, tetradecanol, (*Z*)-7-hexadecenyl acetate, (*Z*)-9-hexadecenyl acetate, and (*Z*)-11-hexadecanol. No (*Z*)-9-tetradecenal was present. Studies indicated that titers of the compounds increased rapidly during the 36 hr after adult eclosion and then leveled off, being maintained at high levels until released when the glandular hairs were exposed. During exposure of the hair pencils substantial amounts of the compounds were released. In vivo application of 500 ng of suspensions of (*Z*)-11-hexadecenyl acetate, (*E*)-11-hexadecenyl acetate, (*Z*)-11-tetradecenyl acetate, or (*E*)-11-tetradecenyl acetate in dimethyl sulfoxide to the surface of the denuded hairpencil gland showed that biosynthesis proceeds to the alcohol via the acetate.

Key Words—*Heliothis virescens*, Lepidoptera, Noctuidae, hairpencil glands, male-produced compounds, hexadecanyl acetate, hexadecanol, (*Z*)-11-hexadecenyl acetate, octadecanyl acetate, octadecanol, tetradecanoic acid, hexadecanoic acid, octadecanoic acid.

INTRODUCTION

Considerable research has been conducted on pheromone chemistry and on the behavior associated with sex pheromones produced by females of Lepidoptera as well as on the morphology of the glands that produce these compounds (Tamaki, 1986). However, substantially fewer studies have been centered on male-produced pheromones. Studies on the chemistry and both intra- and intersexual behavior associated with pheromones produced by males of Lepidoptera are of importance because such information is critical to the elucidation of the chemical communication systems of these insects (Tumlinson and Teal, 1982).

Epidermal glands that produce and disseminate the pheromones produced by males are of diverse structure and varied anatomical position. This diversity is exemplified among moth species when considering the eversible tubelike cornemata present on the genital segments of male arctiid moths (Nielsen, 1979), the thoracic brush organs of *Phlogophora* species (Birch, 1970), wing glands of tortricid and phycitid moths (Grant, 1978), and hairpencil glands present on the genital segments of numerous noctuid species.

Variability in structure and anatomical position of the pheromone glands of males is paralleled by the types of chemicals released as pheromone components and by the behavioral roles of these compounds in chemical communication (Tamaki, 1986). Chemicals released as pheromone components include such different molecules as benzaldehyde (Clearwater, 1972), methyl-jasmonate (Baker et al., 1981), and (Z,Z,Z)-3,6,9-heneicosatriene (Heath et al., 1987). Functions of pheromones released by males range from the attraction of females (Dahm et al., 1971; Baker et al., 1981), to inducing female quiescence, and inhibition of the approach of other males (Clearwater, 1972; Hirai et al., 1978). Unfortunately, neither the structures nor functions of pheromones released by males of the majority of moth species have been described.

Little work has been conducted on the pheromone produced by the hairpencil gland of males of *Heliothis virescens* (F.), although various functional roles have been ascribed. For example, Hendricks and Shaver (1975) suggested that the pheromone stopped females from calling. Alternatively, laboratory flight tunnel studies of mating behavior caused Teal et al. (1981) to hypothesize that the pheromone caused females to accept courting males. Recently, Jacobson et al. (1984) reported that (Z)-9-tetradecenal (Z9-14:Al) was the pheromone produced by males of *H. virescens*. The production of milligram amounts of this compound was of interest because the corresponding alcohol was not identified. This alcohol is present in extracts of the pheromone gland of females (Teal et al., 1986) and is converted to Z9-14:Al, a critical component of the pheromone blend released by females (Teal and Tumlinson, 1986; Vetter and Baker, 1983). This suggested that males and females of *H. virescens* employ different methods of pheromone biosynthesis.

For the above reasons, we investigated the chemicals produced in the hair-pencil glands of males of *H. virescens*. The following reports the identification of a blend of components from these glands and discusses the results of in vivo studies on biosynthesis of these compounds. We also describe the structure of the hair-pencil gland and discuss the possible mechanism of storage of the compounds identified. A subsequent paper will discuss the behavioral role of the pheromone released from the hair-pencil gland in the reproductive behavior of *H. virescens*.

METHODS AND MATERIALS

General. *Heliothis virescens* used in this study were obtained as pupae from the Bioenvironmental Insect Control Laboratory, USDA, ARS, Stoneville, Mississippi. Pupae were sexed and maintained under a reversed 16:8 light-dark cycle at 25°C and 55% relative humidity. Newly emerged adults were transferred daily to 30 × 30 × 30-cm clear plastic cages and were provided with a 10% sugar solution soaked onto cotton.

Anatomical Studies. The terminal abdominal segments, which include the paired hair-pencil glands, one at the base of each clasper, were everted by applying pressure to the anterior abdominal segments. Glutaraldehyde fixative (2% in phosphate buffer pH 7.2) was then injected into the terminal abdominal segments. After 10 min the hair-pencil glands were removed from the abdomen and placed in a vial that contained fixative plus two drops of Photoflow to reduce surface tension. The vial was placed under vacuum at 4°C for 14 hr. Material was postfixed in 2% OsO₄ in phosphate buffer (4 hr) prior to dehydration in ethanol and propylene oxide. Some tissue was then embedded in Spurr's resin and sectioned at 0.5–1.5 μm using glass knives. Sections were mounted in series on gelatin-coated slides and stained using methylene blue in 2% borax (Teal and Philogène, 1980). The remaining tissue was dried in a desiccator that contained granular SiO₂ prior to mounting on stubs using silver conductive paint. These samples were then sputter coated with gold. Scanning electron microscopy was performed using a Cambridge Stereoscan Mark IIA operated at 10 kV.

Isolation and Identification. In initial studies, rinses of the whole male hair-pencil gland complex, including the elongate glandular hairs and cellular portion, were obtained by removing the abdominal segments of males during the 4th to 6th hr of the dark period of the second complete scotophase after emergence. This was accomplished using forceps by applying pressure to the anterior abdominal segments and dipping the exposed gland into 200 μl of iso-octane (Fisher 99% mole) for ca. 30 sec. Up to 25 rinses were collected in each sample, and the extracts were analyzed without concentrating the iso-octane. Extracts of the elongate hairs associated with the hair-pencil gland were

prepared by cutting the hairs carefully from the surface of the gland, leaving a short portion attached and placing the hairs in a 0.5-ml conical microvial. The hairs were then rinsed with 10 μ l of iso-octane that contained the appropriate internal standards per male equivalent (ME).

Two methods were used to collect the compounds volatilized from the hair-pencil glands. In the first method, individual males were placed in an enlarged holding apparatus similar to that described by Teal et al. (1986). This apparatus was connected to another smaller chamber that contained a rubber septum impregnated with 1 mg of the six-component pheromone blend released by females (Teal et al., 1986). Purified air was passed over the lure and into the chamber holding the males. Volatiles released during hair-pencil displays were collected on charcoal microentrainment filters and were recovered in a small amount of dichloromethane and iso-octane as described elsewhere (Teal et al., 1986). In the second method of volatile collection, the hairpencils were everted using forceps, as described earlier, under a stream of dried N₂ (250 ml/min) and above a conical microvial that contained 250 μ l of iso-octane for 30 sec so that the N₂ would carry volatiles into the solvent. The volatiles from the hair-pencil scales of up to 50 insects were collected in the same sample, and the solvent was never concentrated to less than 25 μ l under the N₂ stream.

Prior to methanolysis, the iso-octane was evaporated completely under N₂ from extracts of the glandular hairs of groups of five males or volatile collections obtained from groups of 10 males. Methyl esters of acids present in the hair-pencil pheromone were formed by acid methanolysis (Bjostad and Roelofs, 1984) and by methanolysis using boron trifluoride in methanol (Morrison and Smith, 1964).

Biosynthesis. Studies on age-related production of the identified compounds were conducted using pharate adults that had been removed from the pupal case manually; adult males sampled after emergence but prior to wing expansion; insects that had just completed expansion of the wings; insects sampled 2, 4, and 6 hr after wing expansion; and adult insects 12, 24, 36, and 48 hr old. Studies on the effect of time of day on pheromone production were conducted using insects sampled during the peak of reproductive activity on the second scotophase after adult emergence and on insects sampled 12 hr later during the next light cycle. Studies on depletion of the compounds from the gland were conducted by flying males in a laboratory wind tunnel. The six-component aldehyde pheromone blend identified as the sex pheromone released by females (Teal et al., 1986) was used as a lure. Males were allowed to land and fully expose their hairpencils during a 5-min test period. Only males that fully exposed the hairpencils and attempted copulation numerous times at the source of the female sex pheromone were sampled. In all of these cases the whole hair-pencil gland complex of individual males was everted, removed, and extracted for 30 sec in 30 μ l of iso-octane containing 10 ng of the appropriate

internal standard immediately after testing in the flight tunnel. The volume of the extract was then reduced to ca. 5 μ l and analyzed.

In vivo studies on biosynthesis of the compounds were conducted using glands that were clamped in a fully exposed position with a smooth jawed alligator clip. The glandular hairs of some preparations then were removed carefully with forceps thus exposing the actual gland. The hairs that had been removed were placed in a conical microvial. Other preparations were left intact. Test alcohols or acetates were then applied, with a 1- μ l syringe at a concentration of 500 ng in 1 μ l of dimethyl sulfoxide (DMSO), to denuded gland surfaces, the removed hairs, or the intact preparations. Treated and DMSO control preparations were then incubated for 1 hr at 25°C prior to extraction as described for the individual glands and gas chromatographic analysis. Experiments were conducted during the reproductive period (ca. 4 hr after dark) and 12 hr later during the light period.

Chemical Analysis. Chromatographic analyses were conducted using Hewlett Packard (Avondale, Pennsylvania) 5792 and 5890 gas chromatographs (GC) equipped with splitless and cool on-column capillary injectors and flame-ionization detectors. The detectors of the 5792 GC were interfaced to a Hewlett Packard 3390 reporting integrator. Data from the 5890 GC were acquired at a rate of 20 points/sec through a Chromadapt interface and Adalab data acquisition system (Interactive Microware, Inc., State College, Pennsylvania) and processed using an Apple IIe computer with Chromatohart software. Fused silica capillary columns used routinely for GC analysis included a 15-m \times 0.25-mm (ID) DB 225 (J & W Scientific, Folsom, California), a 30-m \times 0.25-mm (ID) SPB1 (Supelco), and a 30-m \times 0.25-m (id) Supelcowax 10 (Supelco, Bellefonte, Pennsylvania). Only the DB 225 column was used in conjunction with the cool on-column injector. In this case the initial temperature of 80°C was maintained for 0.5 min and then increased at 15°C/min to a final temperature of 135°C. Conditions of chromatography when using the splitless injectors were as follows: initial temperature, 80°C for 1 min; splitless purge at 0.5 min; temperature increase at 25°C/min to 165°C (Supelcowax 10) or 180°C (SPB1). Hydrogen was used as the carrier gas at a linear flow velocity of 38 cm/sec. The primary saturated acetates of tridecanol and pentadecanol (10 ng each) were used as internal standards for both synthetic and natural product samples and were used to calculate relative retention indices and to quantitate the amounts of compounds present in natural-product samples. Further GC confirmation was obtained by cochromatography of the natural product samples with the individual synthetic compounds on all three columns.

Electron impact (EI) and chemical ionization (CI) mass spectra (MS) were obtained using VG1212F (VG Instruments, Toronto, Ontario) and Nermag R10-10 (Delsi Instruments, Fairfield, New Jersey) instruments interfaced to Hewlett Packard 5792 GCs equipped with cool on-column capillary injectors. Helium

was used as the carrier gas at a linear flow velocity of 18 cm/sec. Both isobutane and methane were used as ionization gases. In addition to positive ion CI-MS we also obtained negative ion (methane) spectra for extracts of the hairpencil scales. Samples were chromatographed on both the SPB1 column used in GC studies and a 50-m \times 0.25-mm (id) DB5 (J & W) column under the following conditions: initial temperature, 80°C; temperature program, 20°C/min after 1 min; final temperature, 225°C. The retention times and fragmentation patterns of both the synthetic compounds and those present in natural products were compared. All synthetic chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri).

RESULTS

Structure of Hairpencil Gland. The modified hairs that make up the cuticular structures of the hairpencil gland are elongate and distinct from the flattened structure of nonspecialized body scales (Figure 1A and B). The glandular hairs have rows of circular pores separated by cuticular ridges extending their length (Figure 1C and D). Pores communicate with a duct in the interior of the structure (Figure 1E and F). The density of the pores diminishes toward the distal tip (Figure 1D).

The cellular units in direct communication with the hairs are trichogen cells having closely associated tormogen and unmodified epidermal cells (Figure 1F and G). Nuclei of the trichogen cells are situated in the basal cell area, are well defined, and of lobulate structure. The central and apical areas of these cells are packed with vacuoles and osmiophilic droplets of similar size and structure to the vacuoles. The vacuoles are assumed to have contained lipid that was extracted during fixation and dehydration because traces of lipid were detected in some vacuoles. The apical cell membrane is involuted, leaving a large space that communicated with the duct of the hair associated with each cell (Figure 1G).

Isolation and Identification. Initial GC analyses obtained on three different capillary columns of one male equivalent (ME) ($N = 25$) of the whole gland extracts indicated the presence of three peaks that were present in a consistent ratio. These peaks had retention indices corresponding to hexadecanol (16:OH), hexadecanyl acetate (16:Ac) and (*Z*)-11-hexadecenyl acetate (Z11-16:Ac). In addition, peaks having retention times corresponding to octadecanol (18:OH) and octadecanyl acetate (18:Ac) were present, but the amounts varied substantially. Three other peaks also were present in chromatograms obtained using the SPB1 column. This column was capable of chromatographing free fatty acids, although peak shape was poor. These peaks had both relative retention indices and peak shapes that suggested that they were tetradecanoic (14:Acid), hexadecanoic (16:Acid), and octadecanoic (18:Acid) acids. Methanolysis of the extracts and subsequent chromatography on the three capillary columns indi-

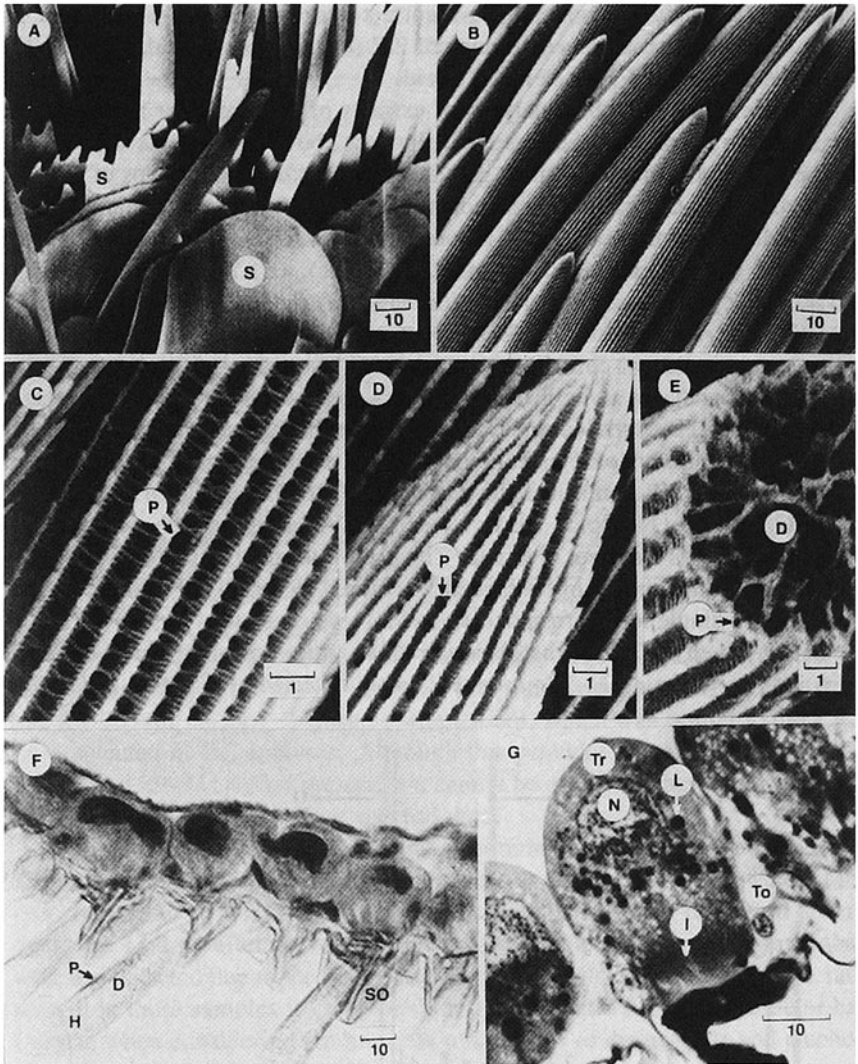


FIG. 1. Scanning electron micrographs and micrographs of hairpencil glands and scales: (A) unmodified scales of male genitalia adjacent to hairpencil gland (S); (B) elongate hairs of the hairpencil gland modified for dissemination of chemicals; (C) pores (P) and ridges along surface of the hair; (D) tip of the hair showing reduction in pore size and number (P); (E) section through tip of hair showing pores in cuticle (P) and internal ducts (D); (F) section through hairpencil gland complex; H = hair, D = duct of hair, P = pores in scale, SO = socket; (G) section through trichogen gland cell (Tr), N = nucleus, L = lipid droplet, I = infolds in apical cell membrane, To = tormogen cell.

cated that the acid assignments were correct because the methyl esters of the acids were detected at concentrations of 2.7 (± 0.9) ng 14:Acid, 22.2 (± 7.3) ng 16:Acid, and 6.3 (± 1.2) ng 18:Acid.

Gas chromatographic analyses of extracts of the glandular hairs that disseminate the pheromone indicated that all of the above components were present in the same ratio found in whole gland extracts. Extracts of groups of five ME analyzed on all GC columns also contained compounds that had relative retention indices corresponding to tetradecanol (14:OH), tetradecanyl acetate (14:Ac), (Z)-9-tetradecenyl acetate (Z9-14:Ac), (Z)-11-hexadecenol (Z11-16:OH), (Z)-7-hexadecenyl acetate (Z7-16:Ac), and (Z)-9-hexadecenyl acetate (Z9-16:Ac) (Figure 2). The amount of 18:Ac was highly variable, and some extracts did not contain this compound. The ratio and concentrations of these compounds are given in Table 1. Mass spectra (60–400 amu) and relative retention indices of the compounds present in the hair extract of 15–25 ME using both capillary columns coupled with EI, positive ion CI (methane), and CI (isobutane) MS confirmed the presence of 16:OH, Z11-16:Ac, 16:Ac, 18:OH, 18:Ac, 14:Acid, 16:Acid, and 18:Acid. Adequate spectra of the other compounds were not obtained. The use of negative ion CI-MS decreased substantially the amount of each acetate required to obtain spectral data. However, only a single ion, corresponding to M-1, was detected. Alcohols were not detected. When 15 ME of the glandular hair extract was analyzed in this manner, ions representing M-1 ($m/z = 281$) were detected at retention times coin-

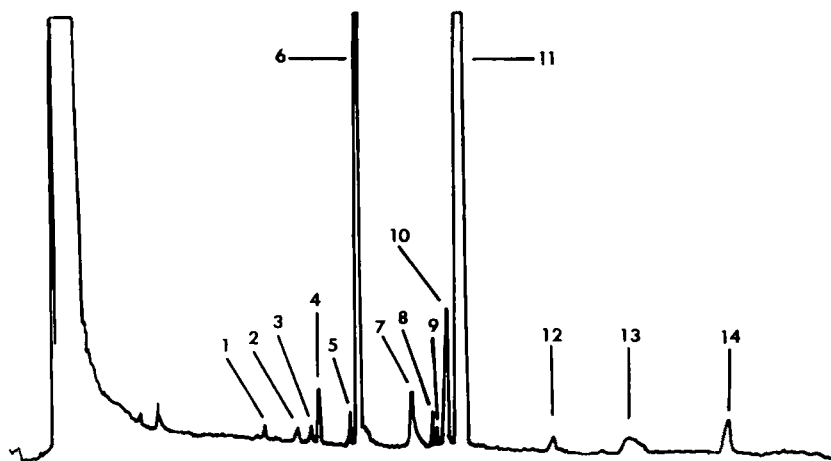


FIG. 2. Chromatogram obtained from analysis of five male equivalents of the extracts of glandular hairs using the SPB1 capillary column. 1 = 14:OH, 2 = 14:Acid, 3 = Z9-14:Ac, 4 = 14:Ac, 5 = Z11-16:OH, 6 = 16:OH, 7 = 16:Acid, 8 = Z7-16:Ac, 9 = Z9-16:Ac, 10 = Z11-16:Ac, 11 = 16:Ac, 12 = 18:OH, 13 = 18:Acid, 14 = 18:Ac.

TABLE 1. COMPOUNDS PRESENT IN HAIRPENCIL GLAND EXTRACTS OF MALES OF *Heliothis virescens* BASED ON GAS CHROMATOGRAPHIC ANALYSIS ($N = 20$).

Compound	Mean amount (ng)	SD	Normalized percentage
14:OH	0.35	0.22	0.16
14:Ac	1.2	0.92	0.56
Z9-14:Ac	0.19	0.09	0.09
14:Acid	2.7	2.1	0.13
16:OH	27.3	15.6	12.85
Z11-16:OH	.27	.25	0.13
16:Ac	212.4	78.4	100.00
Z7-16:Ac	0.52	.40	0.29
Z9-16:Ac	0.18	0.15	0.08
Z11-16:Ac	3.5	1.0	1.16
16:Acid	22.2	7.3	10.36
18:OH	2.5	3.6	1.18
18:Ac	14.2	6.2	6.69
18:Acid	6.3	1.15	2.97

cident with synthetic standards of Z7-16:Ac, Z9-16:Ac, and Z11-16:Ac. The ratio of ion intensities for $m/z = 281$ of the peaks corresponding of Z7-16:Ac and Z9-16:Ac was 2.1:1 (mean of three runs), which was similar to the 2.9:1 ratio detected in GC analyses. Although this provides good evidence that Z7-16:Ac and Z9-16:Ac are present, we cannot be certain of their existence without further and more complete spectral data.

Volatiles collected on the charcoal entrainment filter of the pheromone released by males responding to the female sex pheromone contained an 11:1 ratio of 16:Ac to 16:OH. The greatest amount of 16:Ac recovered from a male was 12.2 ng after six hairpencil exposures. Therefore, minor components were not detected due to the small amount of material collected. The 11:1 ratio present in these samples is comparable to the 9:1 ratio found in glandular hair extracts when considering the recovery efficiencies of the acetates and alcohols from the charcoal filter (Tumlinson et al., 1982). In order to obtain greater quantities of the volatile components, we developed the second technique of volatile collection. GC and GC-mass spectral analysis of volatiles collected in this manner confirmed the presence of 16:Ac and 16:OH. GC analyses also indicated the presence of 14:Ac, Z11-16:Ac, and 18:OH. However, background was too high to obtain useful mass spectra of these compounds. GC and GC-mass spectral analyses of the esterified, volatile samples confirmed the presence of 14:Acid, 16:Acid, and 18:Acid. The ratio of all of the components was consistent with that found in extracts.

Biosynthesis. Studies indicated that the glands of pharate adults did not

contain detectable amounts of either 16:Ac or 16:OH. However, detectable amounts of both compounds were present in extracts of the hairpencil glands of males that had just emerged but had not expanded their wings (Figure 3). The amounts of each compound increased rapidly after adult emergence and peaked at the levels found in isolation identification studies within 36 hr after adult emergence. The titer of these compounds remained high at all times of the light or dark period (Figure 3) provided that the male did not evert the hairpencils. Substantial depletion of both 16:OH and 16:Ac occurred when males exposed their hairpencils numerous times in response to the sex pheromone blend produced by females (Figure 4). The greatest reduction, with respect to control males, was 32-fold for 16:Ac and 27-fold for 16:OH for a male that made eight flights from downwind and exposed his hairpencils several times after each landing. The smallest reduction was fivefold for both 16:Ac and 16:OH, and the majority of insects tested maintained titers of these two compounds that were 8- to 12-fold less than in the control insects. Minor components were not detected in any of the extracts obtained from males that had exposed the hairpencils repeatedly, and the amounts of the acids did not appear to be reduced, although esterified samples were not prepared.

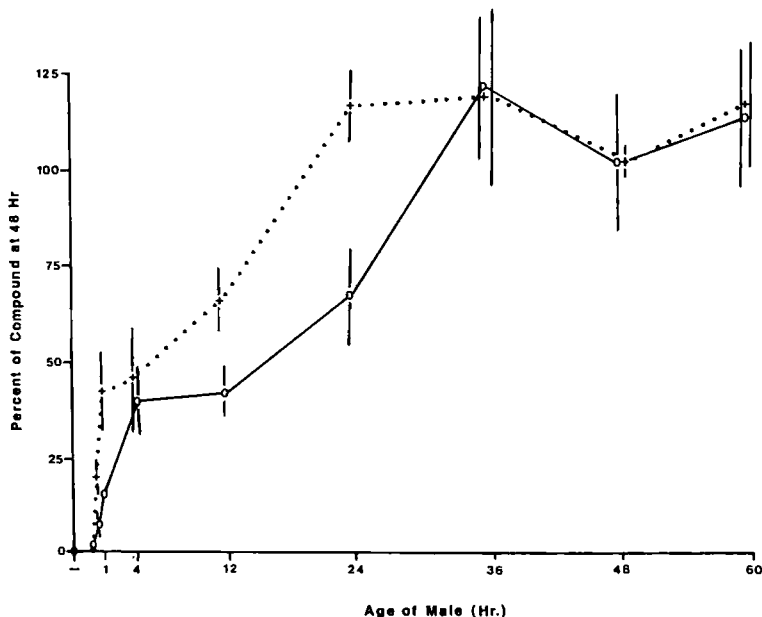


FIG. 3. Titers of 16:Ac (○ — ○) and 16:OH (+ ··· +) in extracts of pheromone glands of males sampled at various ages. Values are normalized to the mean amounts of 16:Ac (212.4 ng) and 16:OH (27.3 ng) present in extracts of males 48 hr old ($N = 5$ for each point).

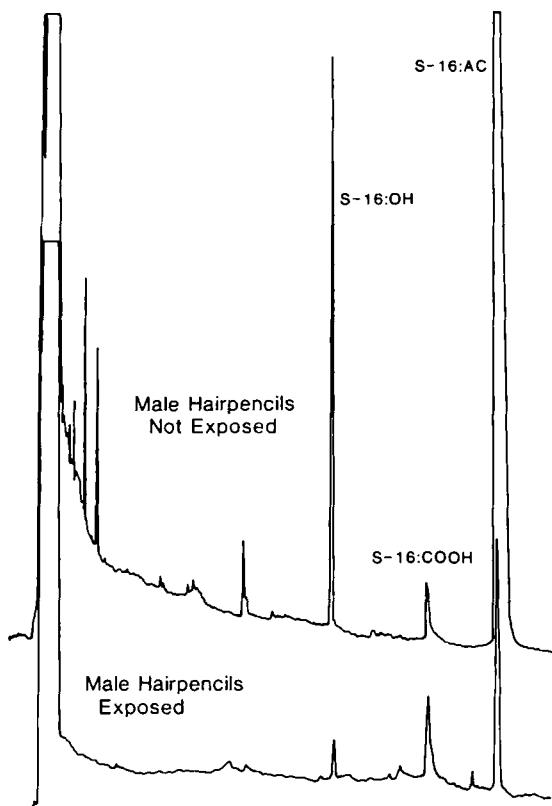


FIG. 4. Comparison of extract obtained from the hairpencil glands of a virgin male *H. virescens* with that obtained from a male that had exposed the hairpencil gland numerous times during four flights from downwind in the flight tunnel. SPB1 column; attenuation = 8× for both chromatograms.

Application of 500 ng of 14:OH or Z11-16:OH to intact hairpencil glands, glands denuded of hairs, and the hairs from denuded glands did not result in changes in the ratios of the corresponding acetates present in extracts. Similarly, other alcohols including (*E*)- and (*Z*)-11-tetradecenol (*E*11-14:OH, Z11-14:OH) or Z9-14:OH were not converted to the corresponding acetates when applied to the gland surface or to either the removed hairs or intact hairs. Scales incubated with 14:Ac, *E*11-14:Ac, or Z9-14:Ac did not convert the acetates to the alcohol analogs. However, substantial amounts of the alcohols were produced when the acetates were applied to the surface of the denuded gland (Figure 5). The conversion showed no specificity for either presence or geometry of double bonds as is indicated by the production of 4.0 (± 1.2) ng more 14:OH

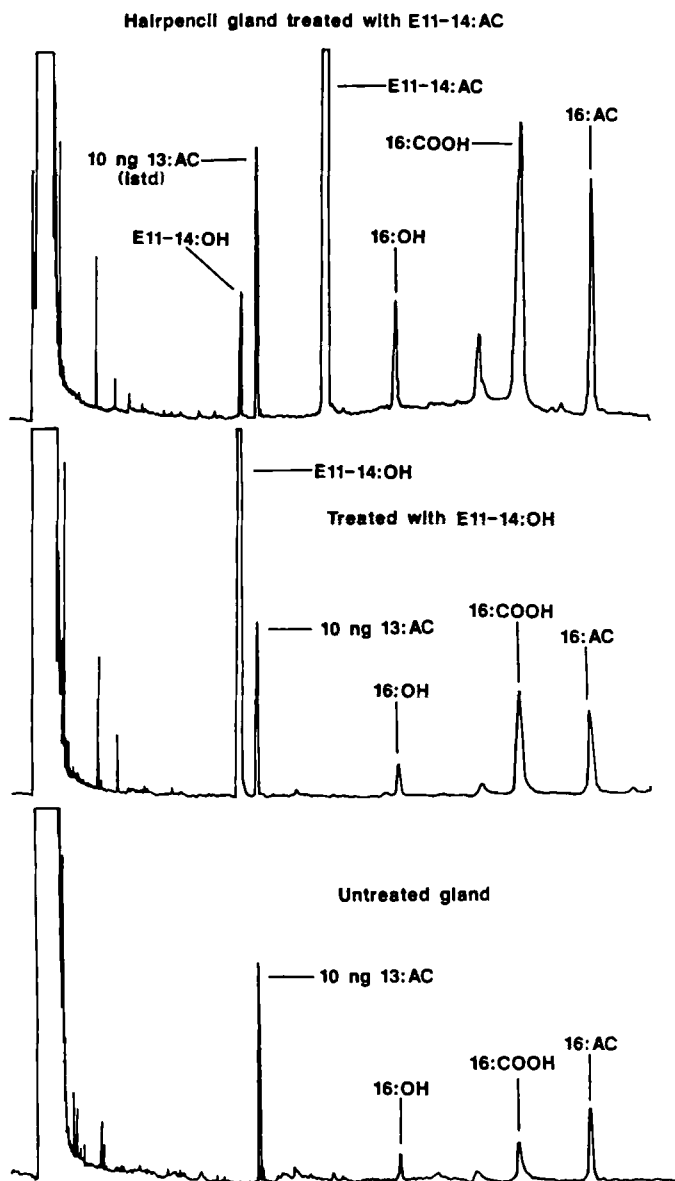


FIG. 5. Comparison of extracts of hairpencil gland (hairs removed) treated with *E11-14:Ac* in DMSO (upper), *E11-14:OH* in DMSO (middle), or untreated (lower) using the SPB1 capillary column.

than was present in glands of untreated preparations ($N = 10$) and $3.7 (\pm 0.7)$ ng ($N = 10$) of $E11-14:Ac$ after a 1-hr incubation. Studies conducted 12 hr after the peak of the reproductive period indicated that the temporal periodicity governing male response to the female pheromone had no effect on the biosynthetic capability of the gland because $3.9 (\pm 0.6)$ ng of $E11-14:OH$ was produced by preparations treated with the corresponding acetate during this period. Similar amounts of $Z9-14:OH$ and $Z11-14:OH$ were produced when glands were treated with the corresponding acetates.

DISCUSSION

Until recently, the components identified as male pheromones, for example, benzaldehyde and 2-phenylethanol (Tamaki, 1986), have had no structural similarity to the components released by females. However, Heath et al. (1988) have documented that males of *Anticarsia gemmatalis* (Hübner) release (*Z,Z,Z*)-3,6,9-heneicosatriene from abdominal scent scales and have shown that this compound is attractive to other males. This compound is one of the pheromone components also produced by females. The identification of the acetates, alcohols, and acids that correspond to the aldehydic components released by *H. virescens* females (Teal et al., 1986) shows a close relationship between pheromone biosynthesis by males and females of this species. This close relationship is also demonstrated by the presence of an acetate esterase that functions to produce alcohol precursors in female glands (Teal and Tumlinson, 1987) as well as in the hairpencil glands where it produces the alcohol components. The function of such esterases is common among females of other moth species, for example *Choristoneura fumiferana* (Clem.) (Morse and Meighen, 1984, 1986), but this is the first report of the function of the esterase in production of pheromone components by males of Lepidoptera.

It is of interest to note that the acetates that correspond to all six of the aldehydes, which comprise the pheromone released by females of *H. virescens*, were found in the hairpencil extracts. However, the ratio of the acetates was very different from that of the aldehydes released by females. For example, the ratio of $16:Ac$ to $Z11-16:Ac$ present in the hairpencil pheromone gland is approximately 68:1, while that of hexadecanal to (*Z*)-11-hexadecenal given off by females is 1:8 (Teal et al., 1986). This indicates that while both sexes possess the enzymes involved in desaturation (see Bjostad and Roelofs, 1983), the enzyme activities are different.

The ratio of the acetates present in greatest amounts to the corresponding alcohols was similar, being about 7:1 for $18:Ac/18:OH$, 8:1 for $16:Ac/16:OH$, 9:1 for $Z11-16:Ac/Z11-16:OH$, and 6:1 for $14:Ac/14:OH$. Therefore, it is probable that the alcohols corresponding to $Z7-16:Ac$, $Z9-16:Ac$,

and Z9-14:Ac are present in the hairpencil gland, but were not identified because of the small amounts present. This hypothesis is supported by our studies on biosynthesis, which have indicated that the acetates are converted to the corresponding alcohols. There also may be a similar correlation between the unsaturated acetates and their corresponding acids, given that the saturated acids that are analogous to 14:Ac, 16:Ac, and 18:Ac were identified. However, this hypothesis cannot be supported at present because, as yet, none of the unsaturated acid analogs have been identified.

The rise in amounts of the alcohol and acetate components present in the hairpencil gland after adult emergence correlates well with reproductive maturity and parallels the increase in production of the sex pheromone produced by females (Shorey et al., 1968). However, while titers of pheromone components drop dramatically after the calling period in the glands of virgin female moths (Pope et al., 1982), the concentration in males remains constant. Therefore, it appears that biosynthesis by males proceeds until a specific titer is reached and then stops with the products being stored, perhaps in the extracellular pocket subtending the hairs. Our data indicate that after depletion of these compounds, during hairpencil exposure, the alcohols and acetates are regenerated at a rate that would ensure that males would have a full titer of these compounds during the next reproductive period. Given that the components released from the hairpencils are important for reproductive success in that they cause females to begin to call and to become receptive to male courtship advances (Teal et al., in preparation), depletion of the compounds during unsuccessful mating attempts would explain why these males are less likely to mate on subsequent attempts during the same reproductive period than are males that have not exposed their hairpencils (Teal et al., 1981).

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FOREST UNDERSTORY BIOMASS HETEROGENEITY Is "Moisture Complex" or Associated Litter the Cause?

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Abstract—Understory biomass heterogeneity under *Platanus occidentalis* L., *Quercus alba* L., *Celtis occidentalis* Pursh., and *Ulmus americana* L. was studied. Soil pH, Ca, Mg, Mn, Cu, NH₄ nitrogen, total N, and soil moisture were significantly different under all dominant species. The pattern of soil moisture levels under different tree species was not consistent from one sampling period to the next. The aboveground biomass of understory vegetation also varied significantly under different tree species and was not correlated with variation in any of the soil properties. Maximum understory biomass gain (340%) from May to September occurred under oak trees, where soil moisture and most nutrients were the lowest. On the other hand, sycamore and hackberry had continuous release of allelochemicals and the smallest understory biomass gain (103%) during the growing season, even though soil under both species had more moisture and nutrients than the soils under white oak. Eliminating all the factors studied leads to the conclusion that organic substances released in the immediate environment of dominant trees and their litter influenced soils and associated herb growth. In each dominant niche system, understory species may have to develop their own system ("micro-niche") to minimize the stresses of competition and allelochemicals.

Key Words—Allelopathy, biomass, mineral elements, soil moisture, deciduous forest

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INTRODUCTION

Vegetation-soil relationships in plant communities are often explained in terms of gradients. Rogers (1983) reported that 10 stands of mesophytic forest sampled were similar with respect to physiography and soil and suggested that many factors (time, weather, floristic composition, frequency, etc.) could be responsible for biomass of understory vegetation. Gauch and Stone (1979) intensively sampled a single 2-ha stand of mixed deciduous forest and found that the main vegetation gradient was due to moisture variability associated with elevation (3 m) differences. However, they also reported that the moisture gradient was accompanied in soil nutrients (pH, N, P, K, Mg, and Mn), texture, and leaf litter. Apparently many soil factors may vary together, forming a complex gradient. McIntosh (1970) stated that in nature, no factor ever varies alone. A "moisture" gradient generally assumes the built-in variation of all available growth-associated factors in a community (Gauch and Stone, 1979).

Parrish and Bazzaz (1976) suggested that species variation in requirements for nutrients can be the cause of niche formation. If different niches are developed by different dominant tree species, then the understory layer under the dominant species would have to develop competitive microniches within the limitation of available resources. Therefore, the biomass of understory species may be influenced under various dominant tree species due to differences in the available resources under them. The niche space cannot be determined by a spectrum of resource types but is due to a spectrum of resource availability (Garbutt and Zangerl, 1983) controlled by the dominant species.

Zinke (1962) studied the patterns of soil properties under single forest trees and found systematic changes in physical and chemical soil properties with varying distances from the tree trunk. Such variation also influenced the ground flora. Similarly, Gersper and Holowaychuck (1971) reported that the soil chemistry pattern under a single forest tree was developed with radial symmetry to the tree. Variations under different species were due to organic litter or to a combination of litter and stem flow (Zinke, 1962; Gersper and Holowaychuck, 1971). Lodhi (1977, 1978b) reported variability in many soil factors under different tree species. Soil pH, Ca, Mg, K, Fe, Zn, Cu, NO_3 , NH_4 , total N, and nitrifying bacteria was found to be different under five different tree species, indicating that tree species exert a strong influence over their own niches.

All these studies reported the variation of factors important for plant growth, but none reported the growth responses of understory vegetation to such factors. Further, there was no indication of the influence of soil moisture on the heterogeneity of nutrient availability and their relation to the understory vegetation under different tree species. Availability of nutrients and their circulation in plants is directly dependent on availability of soil moisture. With these facts in mind, a project was developed to determine the variability of soil moisture

and nutrient conditions in conjunction with understory vegetation growth responses under different tree species in a forest community.

METHODS AND MATERIALS

Location and Description of Study Area. A forest plot was established on the Maryville College campus in St. Louis County, Missouri, ($\approx 39^\circ\text{N}$, 90°W). The vegetation consisted of a mixed bottomland forest in which the important tree species were sycamore (*P. occidentalis*), hackberry (*C. occidentalis*), white oak (*Quercus alba*), red oak (*Q. rubra* L.), and American elm (*U. americana*) with several minor tree species. Important species in the understory were white snakeroot (*Eupatorium rugosum* Houtt.), coralberry (*Symphoricarpos orbiculatus* Moench.), wild rye (*Elymus canadensis* L.), Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.), and downy brome (*Bromus tectorum* L.), with several minor species.

To describe the quantitative growth of understory vegetation under tree species, 10 randomly located 0.25-m² quadrats were clipped in late May and late September under each of four species: American elm, sycamore, hackberry, and white oak. Sampling in May was to estimate the exploitation of nutrient resources at the start of the growing season. The understory growth estimate in late September was considered to be the biomass accumulation of all species and would reflect the utilization and incorporation of available nutrients and moisture.

Physical and Chemical Analyses of Soil. Soil moisture, pH, and nutrient analyses were made to see if the differences in the growth of understory vegetation under American elm, sycamore, hackberry, and white oak correspond to differences in soil chemical and physical properties.

Soil moisture was determined during the months of April, May, June, and August by taking 10 soil samples at the 0- to 15- and 15- to 30-cm levels under American elm, sycamore, hackberry, and white oak at each sampling time. All samples were weighed, oven-dried for 48 hr at 100°C, and reweighed to determine the amount of water lost.

Soil samples for physical and chemical analyses were collected in early April, before the beginning of the growing season, in order to estimate the maximum nutrient amounts available for plant growth. Ten samples were collected at the 0- to 30-cm level under each of the four tree species. The pH was determined with a glass electrode on a 1:4 suspension of soil in aerated distilled water. Potassium, Ca, Fe, Mg, Zn, Mn, and Cu were determined with an atomic absorption spectrophotometer. Ammonium and nitrate were determined with Orion electrodes. Nine of 10 soil samples were combined into three composite samples and analyzed for total N with a Kjeldahl method. Analyses were done

on air-dry soil, but calculations were on the basis of oven-dry weight. Soil moisture was estimated every four weeks (except in July) starting in April.

Data Analysis. Data were analyzed with procedures given by Sokal and Rohlf (1981). Several data sets were found to be not normally distributed, so nonparametric statistical methods were used. The Kolmogorov-Smirnov test was used to test for differences between different sets of the same variable, and Spearman's rank correlation was used to test for association between variables. The word "significant" used below refers to a probability of 0.05 or lower.

RESULTS

Soil pH, Ca, Mg, Mn, Cu, NH_4 , and total N were significantly different under American elm, sycamore, hackberry, and white oak (Table 1). Except from the 15- to 30-cm May sample, soil moisture at the 0- to 15- and 15- to 30-cm levels was also significantly different between all four tree species (Table 2). The variability of soil moisture between different tree species was not consistent from one sampling period to the next. In seven of eight data sets at two depths, white oak had the lowest amounts of soil moisture, while the highest amounts of soil moisture were found under sycamore and hackberry (three data sets each). American elm and oak were each highest in one data set. Soils were nearly saturated at the May and June sampling periods.

The aboveground biomass of understory vegetation varied under different tree species in both sampling periods (Table 3). The biomass of understory vegetation in May was used as an estimate of utilization of the resources avail-

TABLE 1. SOIL CHEMICAL VARIABLES (MEAN \pm SE)^a

Variable	Hackberry (a)	Sycamore (b)	American elm (c)	White oak (d)
Cu	22.2 \pm 1.6 (d)	18.0 \pm 2.0 (d)	18.4 \pm 2.0 (d)	9.1 \pm 0.6 (a, b, c)
Ca	125 \pm 5 (d)	147 \pm 10 (d)	125 \pm 6 (d)	55 \pm 7 (a, b, c)
Fe	425 \pm 3 (b, c, d)	339 \pm 10 (a)	331 \pm 10 (a)	318 \pm 11 (a)
Mn	298 \pm 5 (b, c, d)	273 \pm 4 (a)	279 \pm 11 (a)	283 \pm 4 (a)
Mg	281 \pm 27 (d)	286 \pm 27 (d)	330 \pm 16 (d)	125 \pm 12 (a, b, c)
Zn	18.0 \pm 2.2	23.7 \pm 3.0 (c)	16.6 \pm 1.1 (b)	17.8 \pm 1.9
K	153 \pm 17	136 \pm 7	149 \pm 15	133 \pm 12
NH_4^+	6.1 \pm 0.7 (c)	6.6 \pm 0.6 (c)	2.8 \pm 0.3 (a, b, d)	5.7 \pm 0.5 (c)
NO_3^-	2.8 \pm 0.2	2.9 \pm 0.2	2.4 \pm 0.2	2.4 \pm 0.3
N (total)	1632 \pm 23 (c, d)	1691 \pm 18 (c, d)	1523 \pm 19 (a, b)	1518 \pm 8 (a, b)
H^+ ($\times 10^{-8}$)	22 \pm 2 (c, d)	16 \pm 3 (c, d)	8 \pm 1 (a, b, d)	1507 \pm 180 (a, b, c)

^aValues are $\mu\text{g/g}$ except for H^+ . Letters in parentheses indicate which columns are significantly different ($P < 0.05$).

TABLE 2. SOIL MOISTURE (MEANS \pm SE)^a

Sample	Hackberry (a)	Sycamore (b)	American elm (c)	White oak (d)
April, t ^b	19.4 \pm 1.5 (c, d)	18.6 \pm 1.3 (d)	17.3 \pm 0.7 (a, d)	11.0 \pm 1.0 (a, b, c)
April, s	16.0 \pm 0.6 (d)	16.9 \pm 1.7 (d)	15.2 \pm 0.4 (d)	9.6 \pm 0.6 (a, b, c)
May, t	32.2 \pm 0.9 (c, d)	34.9 \pm 1.7 (c)	29.5 \pm 0.3 (a, d)	35.7 \pm 1.0 (a, b, c)
May, s	29.1 \pm 0.6	29.6 \pm 0.6	29.1 \pm 0.3	28.3 \pm 0.5
June, t	31.8 \pm 0.7 (d)	32.0 \pm 1.6 (d)	29.3 \pm 0.3	28.9 \pm 0.8 (a, b)
June, s	27.6 \pm 0.6 (d)	28.3 \pm 0.6 (d)	28.7 \pm 0.9 (d)	25.2 \pm 0.6 (a, b, c)
August, t	18.8 \pm 1.1 (d)	17.2 \pm 0.8 (d)	18.0 \pm 0.8 (d)	13.2 \pm 0.8 (a, b, c)
August, s	18.4 \pm 1.3 (d)	15.7 \pm 1.0 (d)	16.8 \pm 0.8 (d)	12.3 \pm 0.6 (a, b, c)

^a Values are percent, based on oven-dry weight. Letters indicate which columns are significantly different.

^b t = 0-15 cm, s = 15-30 cm.

TABLE 3. UNDERSTORY BIOMASS UNDER FOUR DOMINANT TREE SPECIES

Species	Biomass (g/m ²)		Change (%)
	May	September	
American elm	61	110	80
Sycamore	35	71	103
Hackberry	29	88	203
White oak	20	88	340

able in April. September understory biomass was considered to be an estimate of the utilization of resources through the growing season. Field soil moisture and vegetation growth were not correlated in most cases (Tables 2 and 3). The understory vegetation under white oak trees, where soil moisture was lowest in both April and August samples at the 0- to 15- and 15- to 30-cm levels, showed the highest biomass increase (340%) from May to September.

DISCUSSION

Usually vegetation growth responses are reported to vary with certain gradients or factors such as soil moisture (Gauch and Stone, 1979; Pickett and Bazzaz, 1976), light, or nutrients (Parrish and Bazzaz, 1976). However, data collected in this study do not show any association of moisture with understory growth under different tree species. Understory biomass response under white oak trees (with lowest moisture amounts) was greatest, contrary to what was

expected. Some of the soil chemical factors were found to differ significantly under the different tree species (Table 1), but no significant correlations were found between any measured chemical or physical soil variable and herb biomass.

Our data indicate that moisture is not the gradient factor associated with the vegetational responses, even when we consider "moisture" as a complex gradient factor that may act as a passive carrier for available nutrients. In this event, maximum growth should associate with maximum soil moisture. Gauch and Stone (1979) reported that understory and dominant vegetation growth pattern was caused mainly by moisture accompanied by gradients in elevation, soil nutrients, pH, texture, and mineral content of leaf litter. Other reports have indicated that understory growth under trees is greatly influenced by tree leaf litter (Jamison, 1942; Zinke, 1962; Gersper and Holowaychuck, 1971; Lodhi, 1977). It should be mentioned that all these reports also found significant differences between soil nutrients under different tree species. However, the reports did not indicate in what way leaf litter quality may have influenced the understory growth.

The function of leaf litter in patterning of vegetation has not been adequately investigated. Gauch and Stone (1979) reported the role of leaf litter nutrients in patterning of vegetation. It seems quite possible that organic substances released from the litter and stem flow may play a major differential stress role in patterning of understory growth under different tree species (Gersper and Holowaychuck, 1971). Levitt (1972) described stress as a condition imposed upon an organism or a population in response to variations in the levels of environmental factors. Organic substances (allelochemicals) may induce physiological conditions in an organism detrimental to its growth. Lauenroth et al. (1978) define stress as a physiological condition that poses a homeostatic threat to the organism. Allelochemicals released by the litter can differentially interact with the herb layer under different tree species. Vegetation under sycamore, with high soil moisture, showed only 103% understory biomass increase from May to September, as compared to 340% increase under white oak trees associated with soils having the lowest amounts of moisture and the most nutrients. Overall, areas under American elm had the most total understory biomass and the least evidence of allelopathy. All three other species had relatively large amounts of allelochemicals under them, but litter decay was at different rates. White oak had a high overwinter retention of allelochemicals, strongly inhibiting early understory growth, but had a lower rate of litter decomposition during the growing season resulting in reduced inhibition. Understory biomass under white oak had the greatest gain (340%), even with the lowest soil moisture and the most nutrients. This is in agreement with a report by Lodhi (1978a) who found that most allelochemicals in white oak are bonded, which results in a very slow rate of release and the highest accumulation in the soil during the early growing

season. Obviously, this slow release of bonded allelochemicals compounded by a slow litter decay rate was not sufficient to inhibit the understory growth rate at the same intensity as in the initial growing period. On the other hand, sycamore and hackberry had more continuous release of allelochemicals, which kept the understory biomass gain to a minimum (103%) during the growing season, even though soils beneath both species had higher soil moisture and nutrients than the white oak soils.

Allelochemic ecological stress may induce a physiological condition that causes interference in plant-water relationships. Colton and Einhellig (1980) designed elaborate experiments to clearly demonstrate the turgor loss, altered leaf diffusive resistance, and water potential changes in plants growing under such allelochemic ecological stress. Altered leaf diffusive resistance and water potential indicate stomatal closure and a probable reduction in availability of CO₂, thus interfering with the photosynthetic mechanisms (Einhellig et al., 1970; Lodhi and Nickell, 1973). Further, Einhellig and Rasmussen (1979) and Colton and Einhellig (1980) demonstrated that allelochemicals and litter containing allelochemicals can significantly lower the chlorophyll content of plants. Glass (1974a, b) reported that many allelochemicals may interfere with nutrient uptake.

Lodhi (1976, 1978a) reported that sycamore, hackberry, and white oak contained many allelochemicals. Further, the leaf decay rate of hackberry was the highest followed by sycamore and white oak. The white oak stand has the maximum (22% of litter) identifiable as oak litter at the last sampling in August, whereas sycamore and hackberry had only 8 and 0%, respectively, in their stands (Lodhi, 1978a). Sycamore contained many free phenolics, which were released in the associated soils, thus increasing the physiological stress on the understory vegetation. White oak contained one free and several bonded allelochemicals in its associated soil and leaves and influenced the understory growth in May more drastically. Later in the season there was a higher growth rate under white oak (Lodhi, 1976, 1978a). Understory biomass under elm trees was the highest at both sampling times.

If the variability in understory biomass under dominant trees was to be attributed (at least partially) to the allelochemicals released by the intact tree vegetation, then the decaying litter should be able to contribute large amounts of inhibitors to the soil before the next growing season. Furthermore, these inhibitors and those in the litter should allelopathically reduce the growth of understory vegetation. Previous work showed that amounts of all identified inhibitors remained high in soils (Table 4) throughout the year, most importantly in the growing season. The decay rate of the litter is such that it maintained the allelopathic potential even in August and reduced the growth by 24% (Table 4). Therefore, there remains no doubt that allelochemicals are directly released from the litter of dominant trees, thus differentially influencing the growth of the understory vegetation as determined by the dominant trees.

TABLE 4. TOTAL AMOUNTS OF ALLELOCHEMICS IN FOREST SOIL AND DECAYING LITTER AND THEIR TOXICITY^a

Sample	Allelochemics, (kg/ha) ^b	Identifiable litter	
		% of total ^c	Toxicity, % of control ^d
January	5676	80	41
April	4927	40	29
August	1722	13	24

^aSummarized from Lodhi (1978a).

^bMore than 50% inhibition in radicle growth caused by 1097 kg/ha of a single allelochemic.

^cAmounts of combined identifiable litter of allelopathic species as percent of total litter.

^dEffects of decaying leaf litter on radicle growth of brome grass. Peat moss was used as control.

It seems logical that the niche system of each dominant tree species significantly differs from the others. Therefore the understory species would have to respond differently in each niche where "physiological conditions" are different. Furthermore, understory plants would have to compete for available resources under the influence of great variability of allelochemic stress caused by the dominant species. Therefore, in each dominant niche system, understory species would have to develop their own system (microniche) to minimize the stresses of both competition and allelochemics.

It is clear that variability in understory growth under different tree species is not the function of a "moisture" factor/gradient even in its complex sense as discussed by Gauch and Stone (1979) and McIntosh (1970). This leaves the litter type and its organic constituents as an alternate driving force in the variability of the understory biomass. We strongly recommend that physiological stresses should be given serious consideration when vegetation responses are measured at the ecosystem, single stand, and/or at the niche level.

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DECREASED SENSITIVITY OF MIXED-FUNCTION OXIDASES FROM *Papilio polyxenes* TO INHIBITORS IN HOST PLANTS

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Abstract—Caterpillars of *Papilio polyxenes*, the black swallowtail, feed on umbellifers that contain both toxic furanocoumarins and methylenedioxyphenyl compounds such as myristicin and safrole. These phytosynergists enhance the toxicity of furanocoumarins by inhibiting mixed-function oxidases (MFOs), the detoxification enzymes responsible for metabolizing furanocoumarins. In model substrate assays, MFOs from *P. polyxenes* are twice as active as MFOs from *Heliothis zea*, a generalist herbivore not adapted to feeding on either furanocoumarins or furanocoumarin/phytosynergist combinations. *P. polyxenes* MFOs are 10 and 46 times less sensitive to inhibition by myristicin and safrole, respectively, than *H. zea* MFOs and eight times less sensitive to inhibition by safrole than MFOs from *Papilio troilus*, a closely related species that does not encounter furanocoumarin/phytosynergist combinations in its diet. Higher MFO activity and decreased sensitivity to MFO inhibitors are important adaptations that allow black swallowtail caterpillars to feed on many umbelliferous plants.

Key Words—Lepidoptera, *Papilio polyxenes*, *Papilio troilus*, Papilionidae, *Heliothis zea*, noctuidae, myristicin, safrole, xanthotoxin, mixed-function oxidase inhibition, methylenedioxyphenyl.

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INTRODUCTION

Caterpillars of *Papilio polyxenes* (Lepidoptera: Papilionidae), the black swallowtail, feed on furanocoumarin-containing plants of the families Umbelliferae and Rutaceae (Berenbaum, 1981). While furanocoumarins are toxic and phototoxic to microbes, vertebrates, and many species of insects (Murray et al., 1982), *P. polyxenes* thrives on diets containing linear furanocoumarins such as xanthotoxin (Berenbaum, 1981). The ability of *P. polyxenes* to tolerate xanthotoxin is due to its rapid metabolism and elimination; xanthotoxin is metabolized much faster in *P. polyxenes* than in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera; Noctuidae), an insect not adapted to feeding on furanocoumarins (Ivie et al., 1983; Bull et al., 1984).

Xanthotoxin detoxification in *P. polyxenes* is largely due to mixed-function oxidases (MFOs), detoxification enzymes located in the midgut (Ivie et al., 1983; Bull et al., 1984). One characteristic of these enzymes is their inhibition by methylenedioxyphenyl-containing (MDP) compounds. In many host plants of *P. polyxenes* (Tyler, 1975), furanocoumarins cooccur with MDP compounds such as myristicin and safrole (Berenbaum, 1983; Neal, 1987). Myristicin and safrole inhibit MFOs and are synergists of some synthetic organic insecticides (Hodgson and Philpot, 1974). They are also phytosynergists (plant compounds that have a synergistic effect on cooccurring toxins) of xanthotoxin (Berenbaum and Neal, 1985; Neal, 1987). When incorporated into an artificial diet, both myristicin and safrole increased the toxicity of xanthotoxin to the corn earworm, *Heliothis zea* (Lepidoptera; Noctuidae), a caterpillar not adapted to feeding on furanocoumarin/phytosynergist combinations (Berenbaum and Neal, 1985; Neal, 1987).

In order to metabolize xanthotoxin rapidly, *P. polyxenes* must contend with the inhibitory effects of MDP compounds. Possible adaptations of *P. polyxenes* for feeding on plants containing both toxins and phytosynergists include increased MFO activity or decreased sensitivity of MFOs to inhibition by MDPs. To investigate these possibilities, the inhibition by MDPs and the activity of MFOs from *P. polyxenes* were compared to MFOs from two other species of Lepidoptera, *Heliothis zea* and *Papilio troilus*. *H. zea*, the corn earworm, was chosen because MDPs are known to have a synergistic effect on the toxicity of xanthotoxin to first-instar larvae (Berenbaum and Neal, 1985; Neal, 1987). Thus, MFOs from this species should be strongly inhibited by MDPs. *P. troilus*, the spicebush swallowtail, feeds on leaves of *Sassafras albidum* (Tyler, 1975). While the roots and the bark contain safrole as the primary oil component, safrole is not detectable in the leaves (Kleber, 1899; Gant and Clebsch, 1975). *Sassafras* is not known to contain furanocoumarins (Murray et al., 1982), and *P. troilus* is rarely reported to feed on plants that contain furanocoumarins (Tyler, 1975; Murray et al., 1982). While closely related to *P. polyxenes*, *P.*

troilus is not exposed to furanocoumarins, although it may have some exposure to safrole in its diet. Thus *P. polyxenes* is expected to be adapted to furanocoumarin/phytosynergist combinations while *P. troilus* and *H. zea* are not.

METHODS AND MATERIALS

Chemicals. $MgCl_2$, $NADP^+$, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase type XI (GDH), phenylmethane sulfonyl fluoride (PMSF), sucrose, EDTA, polyvinylpyrrolidone, xanthotoxin, and Tris HCl were purchased from Sigma Chemical Co. (St. Louis, Missouri); safrole and pentamethylbenzene from Aldrich Chemical Company (Milwaukee, Wisconsin); myristicin from Saber Labs (Morton Grove, Illinois); and *p*-nitroanisole (pNA) and *p*-nitrophenol (pNP) from Eastman Kodak (Rochester, New York).

Insect Rearing. *H. zea* larvae from a laboratory culture maintained at the University of Illinois by Dr. G. Waldbauer (Waldbauer et al., 1984) were reared individually to fourth instar on artificial diet (Berenbaum and Neal, 1985). They were then switched to diet containing 0.2% wet weight pentamethylbenzene, a known inducer of *O*-demethylase activity (Brattsten and Wilkinson, 1973) to facilitate measurement of inhibition. *P. polyxenes* and *P. troilus* larvae were reared from eggs collected from females captured in Illinois. *P. polyxenes* were greenhouse-reared on potted wild parsnip (*Pastinaca sativa*) plants grown from seeds collected in Champaign County, Illinois. *P. troilus* were reared in plastic boxes in a growth chamber on leaves of *Sassafras albidum*.

Midgut Microsome Preparations and Activity. All measurements were made on *H. zea* larvae three days after molting to fifth instar and on *P. polyxenes* and *P. troilus* larvae two to four days after molting to fifth instar. Guts were dissected in ice-cold sucrose medium (0.25 M sucrose; 1 mM EDTA; 1% polyvinylpyrrolidone) containing 2 mM PMSF (Crankshaw et al., 1979) and ground with a Ten Broek homogenizer in sucrose medium with 0.5 mM PMSF. The homogenate was centrifuged at 10,000g for 10 min at 4°C; the supernatant was transferred and centrifuged at 100,000 g for 1 hr at 0°C. The resulting supernatant was discarded and the pellet resuspended in 0.4 M Tris buffer (pH 7.4). *H. zea*, *P. troilus*, and *P. polyxenes* produced 0.36, 0.77, and 1.3 mg microsomal protein per larva, respectively.

A model substrate reaction, *O*-demethylation of pNA, was used for studies of inhibitor kinetics. The kinetics of this reaction have been studied in other insects, and no problems with formation of irreversible complexes or end-product inhibition have been encountered (Hansen and Hodgson, 1971). *O*-Demethylation of pNA was measured by the method of Hansen and Hodgson (1971). Approximately 0.8 mg microsomal protein was incubated for 30 min at 31°C in 10-ml Erlenmeyer flasks containing 0.5 mM $NADP^+$, 2.5 mM G6P, 7.5

mM $MgCl_2$, 0.4 units GDH, 50 mM Tris and pNA, and in a final volume of 1.6 ml. The reaction was stopped by adding 0.4 ml 1 N HCl and pNP extracted into 2 ml dichloromethane. After centrifuging to separate the layers, pNP was extracted from a 1.2-ml aliquot of the dichloromethane with 1.2 ml of 0.5 N NaOH and quantified by measuring absorbance at 400 nm in a Perkin-Elmer dual-beam spectrophotometer. To measure inhibition, inhibitors were added to the incubation mixture in 0.02 ml methyl cellulose prior to adding the microsomes to start the reaction. All reactions were run as duplicates and replicated at least once.

I_{50} values (the concentration of inhibitor required to reduce the reaction rate by half) were determined by linear regression of the overall average percent inhibition on the log of the inhibitor concentration. I_{50} values for inhibition of microsomal *O*-demethylation of pNA were determined using 0.5 mM pNA. The Michaelis constant, K_m , for pNA was determined from plots of reciprocal velocity versus reciprocal concentration. The inhibitor constant, K_i , for safrole and myristicin was determined from the overall mean activities by the method of Dixon (1953). Metabolism of xanthotoxin was determined by the method of Nitao (1987). Fifty micrograms xanthotoxin in acetone solution was added to a 10-ml Erlenmeyer flask and the acetone evaporated. Then 1.2 ml of 0.1 M Tris buffer containing the same NADPH-generating system used for *O*-demethylation was added to the flask and the reaction started by addition of a microsomal suspension to produce a final protein concentration of 0.5 mg/ml. The reaction was terminated after 30 min by the addition of 0.4 ml of 0.1 N HCl. Xanthotoxin was extracted in ethyl acetate and quantified by HPLC as described by Nitao (1987). Dye reagent for protein assay and bovine plasma albumin (Bio-Rad Chemical Division, Richmond, California) were used to determine protein content.

RESULTS

The Michaelis constant, K_m , for binding of pNA by microsomes was approximately 0.4 mM for both *H. zea* and *P. polyxenes*; the V_{max} for *P. polyxenes* (3.8 nmol pNA oxidized/min) was almost twice that for *H. zea* (2.1 nmol pNA oxidized/min) at the same protein concentration (0.5 mg/ml) (Figure 1). In other words, the MFOs from both species have similar affinities for the model substrate, but *P. polyxenes* metabolizes the substrate at almost twice the rate as *H. zea*. Too few larvae were available to measure these parameters in *P. troilus*.

Larger differences among species in the inhibition of *O*-demethylase activity by myristicin and safrole were noted. For 50% inhibition of *O*-demethylation (I_{50}), 2 μ M myristicin was required for *H. zea*, and 92 μ M myristicin was

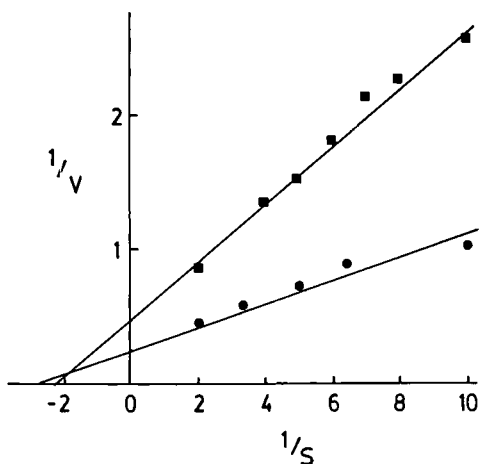


FIG. 1. Double reciprocal plot of *O*-demethylation of *p*-nitroanisole by microsomes from *Heliothis zea* (■) and *Papilio polyxenes* (●). $1/S$ = substrate concentration in $1/\text{mM}$; $1/V$ = velocity in min/nmol . K_m for both *H. zea* and *P. polyxenes* is 0.4 mM . V_{max} is $2.1 \text{ nmol}/\text{min}$ for *H. zea* and $3.8 \text{ nmol}/\text{min}$ for *P. polyxenes*.

required for *P. polyxenes* (Figure 2). The inhibitor constant, K_i , for the dissociation of the enzyme inhibitor complex was $1 \mu\text{M}$ myristicin for *H. zea* and $104 \mu\text{M}$ myristicin for *P. polyxenes*. In *P. polyxenes*, the myristicin concentration required for 50% inhibition of xanthotoxin metabolism ($101 \mu\text{M}$) is similar

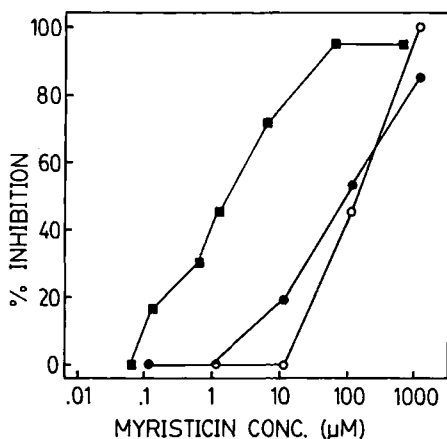


FIG. 2. Percent inhibition versus myristicin concentration for *O*-demethylation of *p*-nitroanisole by microsomes from *Heliothis zea* (■) and *Papilio polyxenes* (●) and metabolism of xanthotoxin by microsomes from *P. polyxenes* (○).

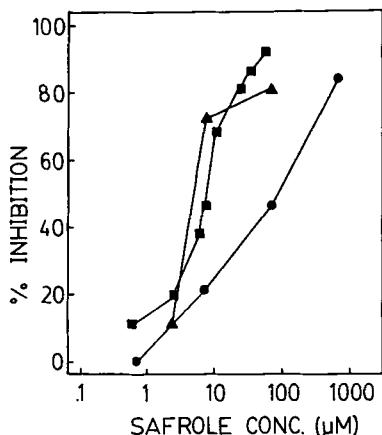


FIG. 3. Percent inhibition versus safrole concentration for *O*-demethylation of *p*-nitroanisole by microsomes from *Heliothis zea* (■), *Papilio polyxenes* (●) and *Papilio troilus* (▲).

to that required for *p*NA *O*-demethylation (92 μ M) (Figure 2). K_i was not determined for inhibition of xanthotoxin metabolism by *P. polyxenes*. Metabolism of xanthotoxin by *H. zea* was too slow to detect inhibitory effects in the 30-min assay.

The I_{50} s for inhibition of *p*NA *O*-demethylation by safrole were 6 μ M with *H. zea* MFOs, 8 μ M for *P. troilus* MFOs, and 64 μ M for *P. polyxenes* MFOs (Figure 3). The inhibitor constant, K_i , for the dissociation of the enzyme inhibitor complex was 2 μ M safrole for *H. zea* and 60 μ M safrole for *P. polyxenes*.

DISCUSSION

There are two factors that allow *P. polyxenes* to feed on furanocoumarin/phytosynergist combinations. The first is more rapid metabolism of substrates by *P. polyxenes*. The V_{max} for *O*-demethylation of *p*NA is greater for *P. polyxenes* than for *H. zea*. This difference could be attributable either to a larger amount of *O*-demethylase or to a more active *O*-demethylase in *P. polyxenes* on a per milligram midgut microsomal protein basis. This high activity extends to other substrates as well; in comparison with *Spodoptera frugiperda*, *P. polyxenes* metabolizes xanthotoxin six times faster (Bull et al., 1984).

Among insect species, differences in rates of MFO activity spanning several orders of magnitude have been reported (Krieger et al., 1971; Rose, 1985), and attempts have been made to correlate MFO activity with the degree of host-plant specialization (Krieger et al., 1971) or the presence of certain classes of

allelochemicals such as monoterpenes in the host plants (Rose, 1985). Many host plants of *P. polyxenes* contain monoterpenes, and MFOs may be responsible for their detoxification. However, a causal link between monoterpenes and high constitutive MFO activity has not been established. Another factor necessitating high MFO activity in *P. polyxenes* is the need to metabolize a toxin in the presence of an MFO inhibitor in the diet. The majority of host plants (Berenbaum, 1981) contain furanocoumarins (Murray et al., 1982), and many of these plants contain MFO inhibitors (Berenbaum and Neal, 1985, 1986). Very high MFO activity has also been noted in *Papilio cresphontes* (Neal, 1987), *P. aegaeus*, and *P. anactus* (Rose, 1985), papilionids that also feed on plants containing furanocoumarins and MDP compounds. Thus, rapid substrate metabolism appears to be an adaptation of papilionids that feed on plants containing furanocoumarin/phytosynergist combinations.

The second factor that allows *P. polyxenes* to feed on furanocoumarin/phytosynergist combinations is its decreased sensitivity to inhibition by MDP-containing phytosynergists such as myristicin and safrole. *P. polyxenes* is 46-fold and 10-fold less sensitive than *H. zea* to MFO inhibition by myristicin and safrole, respectively and eight-fold less sensitive to MFO inhibition by safrole than *P. troilus*. This reduced sensitivity applies to other inhibitors as well. Bull et al. (1986) compared aldrin epoxidase activity in *P. polyxenes* and *Spodoptera frugiperda* in the presence of piperonyl butoxide (PB), a synthetic MDP-containing insecticide synergist. From their data can be calculated an I_{50} of 48 μM PB for *P. polyxenes*, a value five times greater than the calculated value of 8.5 μM for *S. frugiperda*.

The exact mechanism for reduced sensitivity in *P. polyxenes* is unknown. MDP compounds such as myristicin are partially competitive inhibitors of MFOs and can form a noncompetitive inhibitor complex with the enzyme (Hodgson and Philpot, 1974). Reduced sensitivity to MDP inhibitors could result from either more rapid metabolism and elimination of the MDP substituent from the enzyme or lower affinity for MDPs.

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INFLUENCE OF DIEL RHYTHM AND BRAIN HORMONE ON PHEROMONE PRODUCTION IN TWO LEPIDOPTERAN SPECIES

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Abstract—*Heliothis armigera* and *Spodoptera littoralis* females produced sex pheromone during their scotophase. Maximum levels of pheromone in *Heliothis armigera* were extracted from 2- to 3-day-old females during their 4.5-hr scotophase. On ligation between the head and the thorax of both species, normal pheromone production was inhibited. Homogenates of brains (consisting of brains, subesophageal ganglia, corpora cardiaca, and corpora allata) from both species induced a normal level of pheromone production by ligated females. Moreover, in *S. littoralis*, female brain hormone induced the production of the two components (*Z*)-9- and (*Z, E*)-9,11-tetradecenyl acetate tested by gas chromatography. Partial separation of brain homogenates, using low pressure C18-silica reversed-phase columns, produced a stable and active hormone source that gave a dose-dependent response. This hormone was found in brain extracts of both male and female *H. armigera* and *S. littoralis* females. In addition, *S. littoralis* induced pheromone production by *H. armigera* ligated females.

Key Words—Hormonal control, sex pheromone, *Heliothis armigera*, *Spodoptera littoralis*, Lepidoptera, Noctuidae.

INTRODUCTION

Most insects are sexually active during specific times of the day, and the production and release of sex pheromone appears to operate on a circadian cycle (Baker and Cardé, 1979; Cardé and Webster, 1981; Raina et al., 1986). The translation of physiological and environmental signals to the production of sex pheromone has been shown to be mediated by the endocrine system in females

of 12 species (Blomquist and Dillwith, 1983). Most of the early studies used behavioral bioassay techniques, and it was not possible to determine whether the synthesis and/or release of sex pheromone components was influenced by hormones. Using a direct chemical determination of pheromone titer, evidence is now accumulating indicating that pheromone production is under hormonal control in *Heliothis zea* (Noctuidae) (Raina and Klun, 1984a, b), in *Chilo suppressalis* (Pyralidae) (Ohguchi et al., 1985), and possibly in *Platynota stultana* (Tortricidae) (Webster and Cardé, 1984). Recently a pheromone biosynthesis activating neuropeptide (PBAN) has been isolated and purified from brain complexes of *Heliothis zea* (Jaffe et al., 1986; Raina et al., 1987). In the present study, we report on further evidence of hormonal control in two other species of Lepidoptera showing that the hormone may be widely distributed in this order.

METHODS AND MATERIALS

Insect Colonies. A colony of *Spodoptera littoralis* was started from several collections of 70–100 insects from the field during their larval feeding stage. Larvae were raised on the following diet: 500 g ground beans, 200 g alfalfa pellets, 75 g bactoagar, 16 g methyl paraben, 16 g ascorbic acid, 16 ml formaldehyde (40%), 5 g sorbic acid, 160 g yeast extract, 3 liters water. Larvae were kept in groups of 5–10 in 100-ml plastic jars (6.5 × 4.0 cm) and maintained in an incubator at 25°C with a 14:10 light–dark cycle. Pupae were removed from the jars and treated in 0.001% formaldehyde solution for 20 min to prevent viral infection. Female and male pupae were separated and placed in 24 × 24 × 13 cm animal cages (Techniplast) adapted by us for insects. Cages containing female pupae (max 60 pupae/cage) were kept in a separate incubator under the above conditions. Similarly a colony of *H. armigera* was started from several field collections of 200 or more insects. Field-collected larvae (from beans, chickpeas, cotton, maize, and tomatoes) were transferred to artificial diet using the above *Spodoptera* diet. Larvae were kept in groups in plastic containers (20 × 20 × 5 cm) during the first stages. Third-instar larvae were transferred, in pairs, to individual jars (50 ml) to prevent cannibalism. Male and female pupae were separated, and cages containing female pupae were kept in a separate incubator under the same conditions.

Calling Behavior. Calling was observed during the scotophase in the presence of a dim red light. Four groups of 10 females each were observed at 10-min intervals, and the number of females with extruded glands were recorded.

Pheromone Extraction and Separation. Pheromone produced by females was extracted by gently squeezing the abdomen and cutting the extruded 8th and 9th abdominal segments. In this way the pheromone gland in the intersegmental membrane between the two segments was removed. Pheromone glands

were subsequently extracted for 10 min in 100 μ l hexane containing 37.77 ng tridecanyl acetate (Sigma) as the internal standard. A Tracor gas chromatograph with FID and an LDC integrator was used to separate standards and extracts using the following conditions: The initial temperature of 70°C was held for 2 min then increased to 185°C at 10°C/min. The detector temperature was held at 230°C; column inlet temperature was at 225°C. Helium was used as the carrier gas at flow pressure of 22 psi. Separations were performed with a splitless injector on a Carbowax 20 M (Supelco) or a DB 225 (J&W) fused tube glass capillary column.

Preparation of Brain Extracts. Brains (comprising brains, subesophageal ganglia, corpora cardiaca, and corpora allata, and henceforth referred to as brains) were dissected under ice into saline containing: 9.82 g/liter NaCl, 0.48 g/liter KCl, 0.73 g/liter $MgCl_2 \cdot 6H_2O$, 0.84 g/liter NaH_2PO_4 , 0.18 g/liter $NaHCO_3$, and 0.001 g/liter phenol red, at pH 7. Subsequently the tissues were homogenized using 1-ml glass homogenizers. Processing of saline brain homogenates was performed on a low-pressure C18-silica cartridge (Sep-pak, Waters Assoc.), a solvent exchange step in which large proteins and salts are removed from the active sample, rendering a stable extract. All extracts were eluted using 80% methanol after loading, as reported previously (Rafaeli et al., 1985). The methanol was evaporated using a Speedvac concentrator (Savant) and reconstituted in saline before injecting into ligated female abdomens.

In Vivo Bioassay for Hormone Response. The ligation method (Raina and Klun, 1984a) was used to examine the role of brain extracts on pheromone production of both species of Lepidoptera in this study. At the beginning of photophase, 1- to 2-day-old females were ligated between the head and thorax. During the following scotophase 3 hr before maximum pheromone production, the ligated females received an injection of brain homogenate or extract in saline prepared from brains of conspecific males or females. Saline and muscle extract injections were tested as controls. Twenty microliters of the test solution was injected into each female through the intersegmental membrane of the abdomen using a Hamilton syringe.

Statistical Analyses. Statistical analyses were performed on a Vax computer housed at the Agricultural Faculty in Rehovot utilizing SAS statistical software, release 82.2 (SAS, 1982). Treatment means were compared by Student's *t* test to determine significant differences when compared to the controls.

RESULTS AND DISCUSSION

The diel rhythm of pheromone production by *H. armigera* was tested by extracting glands from 2- to 3-day-old females at various time intervals during scotophase. Three-day-old *H. armigera* females produced detectable amounts of Z11-16:Ald, and although a high variability of pheromone concentrations

was observed, maximum levels occurred at 4.5 hr scotophase (Figure 1). The diel rhythm was also evident when calling behavior was observed in 2- to 3-day-old females, and peak activity occurred at 7-8 hr into scotophase (Figure 1), 2-3 hr after maximal levels of pheromone production. A similar phenomenon was observed in the yellow peach moth (Konno, 1986).

Ligation stopped the production of pheromone by females of both *S. litoralis* and *H. armigera* (Tables 1 and 2). In addition, on injecting the abdomens of such ligated females with brain homogenates from males (Table 1) and

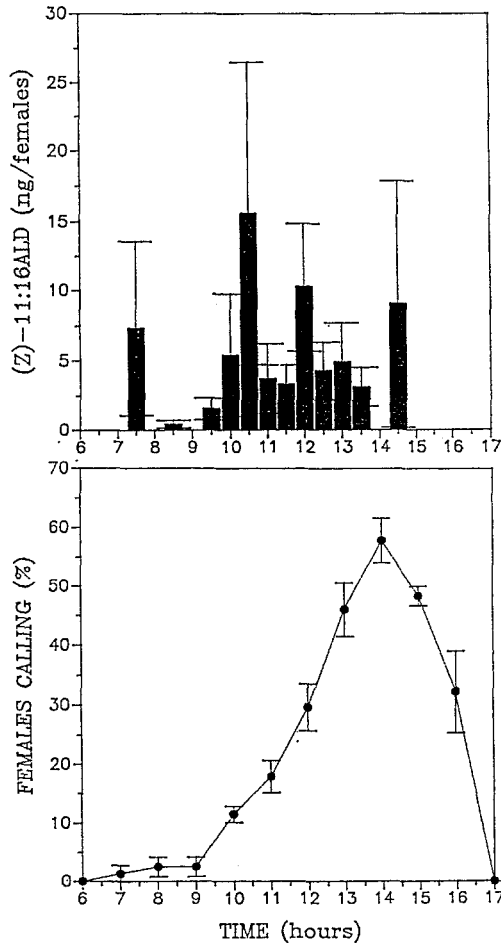


FIG. 1. Pheromone production and calling behavior by 2- to 3-day-old *Heliothis armigera* females at various times during their scotophase (6.30-16.30 hr). Data on calling behavior are presented as means \pm SEM of four replicate experiments.

TABLE 1. INFLUENCE OF MALE AND FEMALE BRAIN HOMOGENATES ON PRODUCTION OF Z11-16: Ald BY 2- AND 3-DAY-OLD *H. armigera* FEMALES IN HOUR 6 OF SCOTOPHASE

Treatment	Z11-16: Ald concentration (ng/gland \pm SEM) ^a	
	2-day-old ♀	3-day-old ♀
Control ligated females	0.00 \pm 0.000b (6)	4.22 \pm 2.027d (5)
Unligated females	9.48 \pm 2.579a (9)	not tested
Ligated females + male brain	8.80 \pm 1.919a (7)	34.67 \pm 12.200c (7)
Ligated females \pm female brain	not tested	60.36 \pm 23.966c (7)

^aNumber of replicates indicated parentheses. Values with different letters are significantly different ($P < 0.001$, 2-day-old $P < 0.01$, 3-day-old, Student's t test).

females (Tables 1 and 2) pheromone production was restored to a level not significantly different from normal female pheromone production, and no significant difference was found between male and female homogenates as was found in *H. zea* (Raina et al., 1984a). Both species responded in the same manner. Clearly, a brain factor is responsible for the control of pheromone production in *H. armigera* and *S. littoralis* females after their ability to do so was inhibited by ligation. This finding supports the existence of a brain hormone that induces pheromone production. Our preliminary results indicate that the hormonal concentration in brains is high at 2 hr into scotophase (11.33 ± 3.67 ng/gland; $N = 9$) and subsequently declines (5.21 ± 4.12 ng/gland; $N = 7$) at 7 hr of scotophase.

Presumably, ligation prevented the timely release of the stimulating hor-

TABLE 2. INFLUENCE OF BRAIN HOMOGENATES ON PRODUCTION OF Z9-14: Ac AND Z,E9,11-14: Ac BY 2-DAY-OLD *Spodoptera littoralis* FEMALES AT HOUR 4 OF SCOTOPHASE

Treatment	Amount (ng/gland \pm SEM) ^a	
	Z9-14: Ac	Z, E9,11-14: Ac
Ligated female control	0.99 \pm 0.990b	0.95 \pm 0.953b (6)
Unligated females	12.31 \pm 2.211a	4.30 \pm 3.543b (7)
Ligated females + male brain (1 brain equiv)	12.80 \pm 4.107a	5.31 \pm 2.380b (6)

^aNumber of replicates indicated in parentheses. Values with different letters are significantly different ($P < 0.001$, Student's t test).

mone from reaching the target tissue. In *H. zea* (Raina and Klun, 1984a, b) and *C. suppressalis* (Ohguchi et al., 1985) ligation or decapitation at later time intervals before peak pheromone production showed a smaller inhibition of pheromone production due to this timely release of the hormone into the blood. It can be seen that *S. littoralis* female production of both pheromone components (*Z*)-9- and (*Z, E*)-9,11-tetradecenyl acetate were stimulated by brain hormone (Table 2). A similar phenomenon was observed in *C. suppressalis*, where levels of (*Z*)-11-hexadecenal and hexadecenal were stimulated by injections of brain extracts (Ohguchi et al., 1985). Thus, where tested, the brain extracts stimulated the production of more than the main pheromone component and, most likely, it can be speculated that the correct pheromone blend is produced.

Fresh saline homogenates were unstable, unless used fresh under ice, and we thus tested the effect of processed brain homogenates. Male and female processed brain homogenates showed an increase in pheromone production in ligated *H. armigera* females that was not significantly different from normal unligated females (Table 3). This increase in pheromone production is similar to that found with fresh brain homogenates (Table 1). A control extract of thoracic muscle was tested and showed no increase in pheromone production.

The response to these brain extracts increases with increasing incubation time after injection (Figure 2) and is dose-dependent (Figure 3), revealing a typical hormonal dose-response relationship.

Purified *S. littoralis* brain extracts were tested on the response of ligated *H. armigera* females. The results showed that *H. armigera* ligated females were stimulated to produce Z11-16:Ald by *S. littoralis* brain hormone to the same extent as stimulation by brain hormone from *H. armigera*. The fact that *S. littoralis* brain hormone was capable of inducing pheromone production by *H. armigera* females, in which the pheromone components differ widely from those

TABLE 3. EFFECT OF *Spodoptera littoralis* FEMALE AND *Heliothis armigera* FEMALE AND MALE PARTIALLY PURIFIED BRAIN HORMONE ON PRODUCTION OF Z11-16:Ald BY 2 TO 3-DAY-OLD *Heliothis armigera* LIGATED FEMALES

Treatment	Z11-16:Ald (ng/gland \pm SEM) ^a
Ligated control females	0.63 \pm 0.63b (6)
Ligated females + thoracic muscle extract (0.66 μ g/female) ^b	0.00 \pm 0.00b (7)
Ligated females + male <i>H. armigera</i> brain (2 equiv)	22.80 \pm 7.84a (5)
Ligated females + female <i>H. armigera</i> brain (2 equiv)	40.10 \pm 14.96a (5)
Ligated females + female <i>S. littoralis</i> brain (2 equiv)	22.30 \pm 9.70a (10)

^aNumber of replicates indicated in parentheses. Values with different letters are significantly different ($P < 0.001$, Student's *t* test).

^b0.66 μ g is equivalent in protein content of 2 brain equivalents.

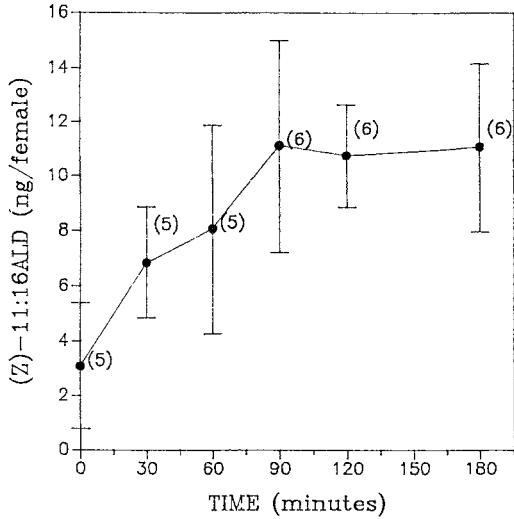


FIG. 2. Effect of incubation time on in vivo Z11-16:Ald production by *Heliothis armigera* females after partially purified brain injection (2 male brain equivalents). The data are presented as the means \pm SEM. Figures in parentheses are the number of observations.

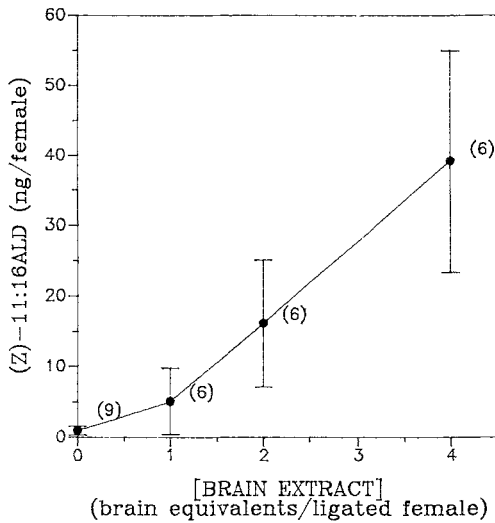


FIG. 3. Effect of increasing doses of partially purified brain hormone from males on in vivo Z11-16:Ald production by ligated *Heliothis armigera* females after 3 hr of incubation. The data are presented as the means \pm SEM. Figures in parentheses are the number of observations.

of *S. littoralis*, supports a possible universal role of a brain hormone in this order, as was suggested by Raina and Klun (1984a, b). This may indicate that hormonal stimulation is effective at least before the desaturation of the pheromone precursor, if we presume a saturated fatty acid as precursor (Bjostad and Roelofs, 1983; Arai et al., 1984; Dunkelblum et al., 1985). It can thus be speculated that the brain hormone induces some general and early step in the biosynthetic pathway involved in pheromone production; however, this awaits further investigation into the biosynthetic pathway of lepidopteran pheromones.

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INTRASPECIFIC VARIATION OF TWO COMPONENTS IN SEX PHEROMONE GLAND OF *Planotortrix* *excessana*¹ SIBLING SPECIES

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Abstract—Two components, (Z)-5- and (Z)-7-tetradecenyl acetates, in the sex pheromone gland of a *Planotortrix excessana* sibling species, were found to vary continuously from a ratio of 3:97 to 71:29 in individual female moths reared from wild larvae collected around New Zealand. Two laboratory colonies were established from populations representing extremes of these ratios. Although both the ratios and the total quantities of the components in individual female moths from both colonies were significantly different from each other, there was a small number of females that overlapped in both ratio and quantity. The variation was confirmed as intraspecific in a field cage experiment with male moths from both populations mating with tethered female moths from both populations.

Key Words—Sex pheromones, *Planotortrix excessana*, Lepidoptera, Tortricidae, leafroller, intraspecific variation, (Z)-5-tetradecenyl acetate, (Z)-7-tetradecenyl acetate.

INTRODUCTION

Female-produced sex pheromones of the Lepidoptera are generally multicomponent blends of chemicals derived from mono- or polyunsaturated long-chain fatty acids (Tamaki, 1985). Common to many species are precisely regulated mixtures of opposite *E* and *Z* geometric isomers that are critical for the specificity of the chemical communication system (Roelofs and Cardé, 1974).

In a study on the redbanded leafroller, *Argyrotaenia velutinana*, Miller and

¹Lepidoptera: Tortricidae: Tortricinae.

Roelofs (1980) found that the ratio of (*E*)- and (*Z*)-11-tetradecenyl acetates (*E*- and *Z*11-14:OAc) in individual female pheromone glands was precisely regulated at 9.1 ± 1.8 (% *E*11-14:OAc \pm SD) and 7.0 ± 1.4 in field-collected and laboratory colony populations respectively. Significant variation from a precisely regulated ratio as above has been used as a way of differentiating closely related, morphologically similar sibling species (Roelofs and Comeau, 1969). In this case, male responses to synthetic lures paralleled the differences in female production.

Although ratios of *E* and *Z* geometric isomers appear to be tightly controlled, significant variation has been noted in the ratios of monounsaturated acetates that are not geometric isomers; these include the summerfruit tortrix moth, *Adoxophyes orana* (Guerin et al., 1986); the turnip moth, *Agrotis segetum* (Löfstedt et al., 1985, 1986); and the larch budmoth, *Zeiraphera diniana* (Guerin et al., 1984). In a recent report, Du et al. (1987) showed that the amount of (*E*)-11-tetradecenyl acetate to its geometric isomer, (*Z*)-11-tetradecenyl acetate, was less variable than the relative amounts of other components in *Yponomeuta padellus*. In species where pheromone component ratios are not precisely regulated, it is important to determine the amount of intraspecific variation within female production and the parallel conspecific male responses, in order to separate this from possible interspecific differences.

Galbreath et al. (1985) reported pheromone differences in the endemic New Zealand leafroller moth pest *Planotortrix excessana* (Walker). Populations collected from Auckland (northern North Island) and Christchurch (mid-coastal South Island) were found to use a mixture of (*Z*)-8-tetradecenyl acetate and tetradecyl acetate. A population from Tokoroa (mid-North Island), however, used two completely different monounsaturated acetates, (*Z*)-5- and (*Z*)-7-tetradecenyl acetates (*Z*5-14:OAc and *Z*7-14:OAc). In a field cage experiment, males of the two populations (Christchurch and Tokoroa) were attracted only to pheromone extracts from females of their own population, leading Galbreath et al. (1985) to suggest that these two populations are sibling species.

This work was extended (Foster et al., 1986) by sampling individual females from a greater number of geographical areas. Within the *Z*5-14:OAc/*Z*7-14:OAc sibling species reported by Galbreath et al. (1985), two distinct groupings of females were observed. One produced ratios of the two components (*Z*5-14:OAc-*Z*7-14:OAc) from 46:54 (not 54:46 as misprinted in Foster et al., 1986) to 70:30, with an average quantity of the two components of 3.5 ng per female, and the other produced ratios from 12:88 to 33:67, with an average quantity of 22 ng per female.

In order to ascertain whether these differences are real, we analyzed a much greater number of female moths collected from various locations, including a number from laboratory colonies, to determine the variation within single populations. Further, to test the biological significance of this variation of female

production for male moths, we developed a rigorous field cage technique in which tethered virgin females from different populations are tested with males of one of the populations to determine whether mating occurs.

METHODS AND MATERIALS

Insects. Insects were collected as larvae from the North, South and Stewart Islands and reared to adult on a synthetic diet (Singh, 1974). Pupae were sexed, and male and female pupae kept separate.

Laboratory colonies of the two populations studied in detail (Waikato, North Island; and Dunedin, South Island), were established by mating groups of three to five males and females each in capped 100-mm-diameter \times 60-mm-high plastic containers. A thin layer of vermiculite was placed on the bottom of the container. Honey solution in a dental wick was available for the moths to feed on. Females oviposited on scored plastic removable liners located inside the containers. Neonate larvae were harvested and reared to adulthood as described in Singh (1974).

The insects were generally maintained at 18°C, but the temperature was changed to 10°C at times throughout the development of the larvae and pupae, to ensure synchronization of the two colonies. For the work reported here, the colonies originating from Waikato and Dunedin were in their fifth and second laboratory generations, respectively.

Females for capillary gas-liquid chromatographic (GLC) analysis were maintained on a reverse light-dark cycle (16 hr light-8 hr dark).

For field cage experiments (see below), pupae were conditioned outside for at least a week prior to emergence, before use.

GLC Analysis. The pheromone glands of individual female moths between 3 and 5 days old were excised 1-2 hr into the scotophase period using fine forceps under a binocular microscope and extracted by allowing to stand in ca. 10 μ l of distilled pentane for approximately 24 hr at ambient temperature.

The gland extracts of individual female moths were analyzed on Pye Unicam 4500 and Varian 3500 gas chromatographs. Both machines were equipped with splitless injection and flame ionization detection. Three fused silica capillary columns were used: a 50-m \times 0.32-mm ID, S.G.E. Corp. (Ringwood, Australia) BP20 (stabilized Carbowax 20 M); a 50-m \times 0.25-mm ID, Quadrex Corp. (New Haven, Connecticut) CPS-1 (bonded SP2340); and a 50-m \times 0.25-mm ID, Silar 10C (Quadrex Corp.). These columns were temperature programmed after a delay of 1 min at the initial temperature as follows: 100-200°C at 4°C/min (BP20 column), 100-180°C at 3°C/min (CPS-1 column), and 60-140°C at 8°C/min then to 180°C at 2°C/min (Silar 10C). Nitrogen was the carrier gas at linear flow rates of 25 cm/sec (BP20 and CPS-1 columns) and 8

cm/sec (Silar 10C). Quantities of chemicals were calculated relative to added tridecanyl acetate internal standard.

Statistical comparisons of pheromone production by individuals of populations were carried out using the Wilcoxon rank-sum test. All differences are reported to $P < 0.05$.

Field Cage Trial. The trial was conducted in two 10-m \times 10-m \times 2.4-m-high field cages, covered with 20 \times 30 mesh fiberglass cloth (Sarlon Reid, Auckland, New Zealand). The two cages were situated approximately 50 m apart and sited at Mt. Albert Research Centre, Auckland.

Twenty-five, 75-cm-long bamboo poles were suspended from the roof of each field cage in a symmetrical 5 \times 5 grid. The base of each pole was located approximately 1 m from the floor of the field cage and just above the plant growth inside the field cage, which consisted of *Griselinia littoralis* (Raoul) (a native host plant of *P. excessana*) and various unidentified weeds.

Three-day-old virgin female moths were tethered by a piece of fine cotton (80 grade), approximately 40 cm long, formed into a noose and placed and tightened around the base of the forewing and hindwing on one side of the moth. The other end of the cotton was wedged into a slit on the bamboo pole. This left the moth free to move around the pole and as free as possible to call and mate. Females were replaced every one or two days. After females had been replaced, their genitalia were dissected under a binocular microscope and the presence or absence of a male spermatophore in the female bursa copulatrix was recorded.

Male moths 2–3 days old were released daily into the field cages, commencing one day prior to the start of the experiment. Male moths inside the cages were left to accumulate during the course of the experiment (10 days).

Tethered females from two populations (Waikato and Dunedin) were placed inside both field cages. Waikato males were released into one cage and Dunedin males into the other cage. In the cage containing Waikato males, a total of 61 Dunedin females and 87 Waikato females were used. In the cage containing the Dunedin males, a total of 38 Dunedin females and 51 Waikato females were used. Totals of 233 Waikato males and 65 Dunedin males were released into the cages.

Analysis of this experiment was conducted using least significant difference tests with percentages transformed to their arcsine value.

RESULTS

The results of analyses of 145 individual females of the Z5-14:OAc/Z7-14:OAc sibling species of *P. excessana* (Galbreath et al., 1985) collected as larvae are summarized in Figure 1. These data show a wide range both of ratios

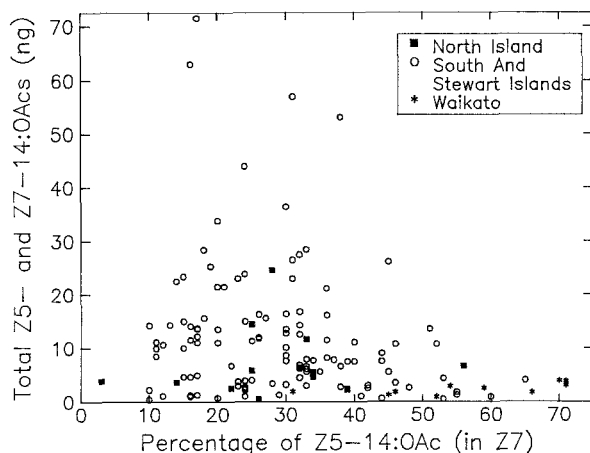


FIG. 1. Plot of total quantity of (Z)-5- and (Z)-7-tetradecenyl acetates (Z5- and Z7-14:OAc) and percentage of Z5-14:OAc in individual feral-collected female *Planotorix excessana*.

of Z5-14:OAc-Z7-14:OAc from 3:97 to 71:29 and quantities of these two chemicals per female from 0.4 ng to 71.5 ng. Quantities of these chemicals greater than 15 ng occurred only with the relatively tight ratios between 14:86 and 45:55, while insects with less than 5 ng had ratios covering the entire range. The mean ratio and quantity were $31:69 \pm 14$ and 10.9 ± 12.1 ng respectively. If the data from wild-collected individuals from the Waikato region (mid-North Island) are ignored (see below), then there are no significant differences in ratio or quantity between North Island and South Island individuals.

Two populations representing the greatest differences, one from Waikato and the other from Dunedin (southern South Island), were selected for further analysis. The wild-collected individuals from these populations show significant differences (Figure 2) in both ratio and quantity. Colonies of these two populations were established, and larger numbers of individual female moths from both populations were analyzed by GLC.

These analyses confirmed the differences between the populations in both ratio and quantity. The Dunedin colony females had a relatively tight range of ratios (Z5-14:OAc-Z7-14:OAc) from 11:89 to 42:58 with a mean of $24:76 \pm 7.7$. The quantity was more variable, ranging from 2.4 ng to a maximum of 59.3 ng with a mean of 13.2 ± 11.2 ng. The Waikato colony females, on the other hand, showed a much wider range of ratios from 23:77 to 81:19, mean $46:54 \pm 17$, but the quantity was less variable, ranging from 0.4 ng to 7.2 ng, mean 2.4 ± 1.6 ng.

It is important to note that, while the two colonies are significantly different

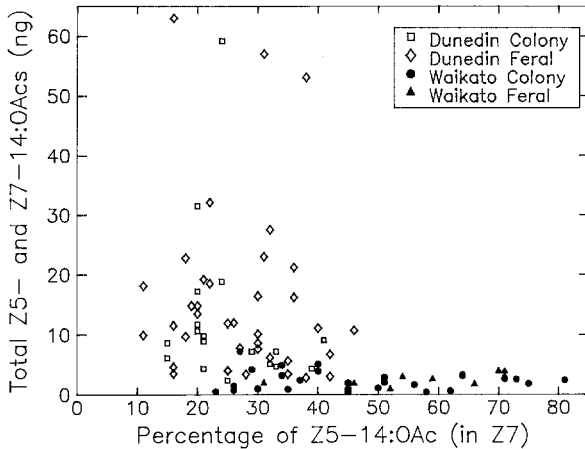


FIG. 2. Plot of total quantity of (Z)-5- and (Z)-7-tetradecenyl acetates (Z5- and Z7-14:OAc) and percentage of Z5-14:OAc in individual female *Planotortrix excessana* from laboratory colonies originally collected from Dunedin and Waikato. Feral-collected individuals from these regions are included for comparison.

in both ratio and quantity of these two components, there is some overlap of these two characters between the two colonies (Figure 2).

In the field cage containing Waikato males, a greater percentage of Dunedin females were mated than the Waikato females (18.0% to 6.9%), although this was not significant ($P > 0.05$). The cage containing Dunedin males gave significantly ($P < 0.05$) greater mating of Dunedin females than Waikato females (28.9% to 9.8%). Although not significant ($P > 0.05$), both mating percentages in the cage containing Dunedin males were higher than in the cage containing Waikato males, despite a much lower density of Dunedin males. Total numbers of mated females of both colonies were almost the same in both cages.

DISCUSSION

Analysis of the sex pheromone produced by female moths of the Z5-14:OAc/Z7-14:OAc *P. excessana* sibling species (Galbreath et al., 1985) shows that the ratio and quantity of two components are highly variable in both wild-collected and laboratory colony individuals.

Our initial observation (Foster et al., 1986) that two discrete groups of ratios and quantities existed, i.e., type B, ratio 46:54 to 70:30, mean quantity 3.5 ng; and type C, ratio 12:88 to 33:67, mean quantity 22 ng, is shown to

be untrue. Sampling of more individuals shows a near continuous range of ratios from 3:97 to 71:29 and a near continuous range of quantities up to 36 ng/female. A few female moths with quantities greater than this were observed, up to 71.5 ng.

However, within these wide ranges there are significant differences between populations. All the wild-collected and colony individuals analyzed from the Waikato population showed a very low quantity of the two components (mean quantity = 2.4 ng, maximum found in an individual = 7.2 ng). Additionally, a very wide range of ratios of the two components were found in these individuals (23:77 to 81:19). The distributions of quantities and ratios in both wild and colony individuals from the Waikato population were significantly different (Figure 2) from those from the Dunedin population that contained on average a much greater quantity of the two components and lower Z5-14:Ac-Z7-14:Ac ratios. However, a number of individuals (both wild-collected and colony-reared) from the two populations, coincided in both quantity and ratio (Figure 2).

The significance of this variation was tested in the field cage experiment. This showed clearly that males of both populations mated with females of both populations under simulated field conditions. Moths of both populations likely completed the whole gamut of male and female behavioral responses through to the transfer of a male spermatophore to the female's bursa copulatrix.

The differences between populations in female production of the two components, Z5-14:OAc and Z7-14:OAc, are therefore probably examples of intraspecific variation and not an interspecific difference, i.e., characters not capable by themselves of conferring reproductive specificity or characters paralleling other significant differences ensuring reproductive specificity of the populations. The two populations used in this study are, however, geographically isolated, and the prospect for direct gene flow between them is extremely unlikely. The wide range of ratios observed in other feral populations throughout New Zealand (Figure 1) is consistent with the above interpretation.

The differences between the populations are nevertheless interesting, particularly as they appear to be heritable and, in the case of the Waikato population at least, have survived through five generations of a laboratory-reared colony. Whether these differences are polymorphic expressions of a gene or gene complex or are environmentally induced polyphenisms (Shapiro, 1976) is unknown and will be the subject of further study.

Previous studies noting intraspecific variation in female pheromone production have not tested the behavioral responses of male moths to females from the different populations. In the case of the turnip moth, the differences noted between populations in female production have been paralleled by differences in attraction of male moths to synthetic blends approximating the observed female pheromone differences (Arn et al., 1983) and in male antennal receptor

frequencies (Löfstedt et al., 1986). In order to differentiate interspecific differences from intraspecific variation in morphologically similar populations, it is important that the behavioral responses of males from different populations to the female moths from the same and different populations are tested. This is particularly necessary where the difference is more difficult to resolve, i.e., between ratios of common components.

The lack of such a behavioral comparison between the different pheromone "dialects" reported by Löfstedt et al., (1986) leaves the true biological significance of this variation unanswered, i.e., whether the differences observed in the French population are representative of intraspecific variation or of interspecific differences from the Swedish, Hungarian, and English populations.

In our field cage experiment, there was a greater bias in mating by males from both populations with the Dunedin females. This may indicate that females of the Dunedin population are more attractive (possibly due to quantity or ratio of chemicals) to males than the Waikato females. This was paralleled by the lower percentages of mating of both Waikato and Dunedin females by the Waikato males, despite a much higher density of males in this cage compared to the cage with Dunedin males. In the absence of further evidence, we are cautious about attaching too much significance to this result, particularly as the overall mating was low. We suggest that the bias may be an artifact of laboratory rearing. At the time of the experiment, the Waikato colony had been in culture for five generations, and the Dunedin colony for only two generations. This or the temperature manipulation used to synchronize the colonies may have resulted in poorer sexual fitness of the Waikato colony.

The technique of using tethered females as a measure of cross-mating offers several advantages over the usual, but less rigorous, method of using virgin females as baits in sticky traps (Sanders et al., 1977). First, all necessary behaviors leading up to copulation must be effectively carried out, including long-range pheromone-mediated flight and any necessary specific male behavioral or chemical responses. Second, the presence of a male spermatophore in the female bursa copulatrix indicates genitalic compatibility, and third, males are not constantly being removed from the environment and therefore fewer are required for the experiment.

The major disadvantage of this method compared to the method using caged female moths in sticky traps is that the responses of two or more different "types" of male moths cannot be compared competitively in the same cage. Additionally, greater numbers of female moths are required for this method.

The variation in pheromone production reported here and in the limited examples noted for other species that do not use mixes of opposite geometric isomers, is substantially greater than the variation reported for species using mixtures of geometric isomers. Until we understand better the mechanisms involved in pheromone production and perception, and how precise ratios and

variable ratios are regulated and perceived in species, the biological importance of pheromone component variation will remain speculative.

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EFFECTS OF PHENOLIC ACIDS ON GERMINATION AND EARLY GROWTH OF HERBACEOUS WOODLAND PLANTS

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Abstract—Four herbaceous plant species from woodland (clearings), *Deschampsia flexuosa*, *Scrophularia nodosa*, *Senecio sylvaticus*, and *Chamaenerion angustifolium*, were tested for their sensitivity to phenolic acids. Seven commonly occurring phenolic compounds were used in a germination experiment in concentrations ranging from 0.01 to 10 mM, i.e., salicylic, *p*-hydroxybenzoic, syringic, caffeic, vanillic, *p*-coumaric, and ferulic acids. Germination was delayed rather than inhibited. Radicle elongation was strongly affected; at lower concentrations stimulatory effects were observed, whereas at high concentrations radicle elongation was severely reduced. Salicylic acid was the most effective phenolic compound, whereas caffeic acid caused no effects. Early growth was studied in more detail in a second experiment with *Deschampsia flexuosa* and *Senecio sylvaticus* and the phenolic acids, ferulic and *p*-coumaric acid. Primary root length, number and length of secondary roots, and dry weight were stimulated at 0.01 mM but were inhibited at 10 mM of both compounds. The results are discussed in view of the allelopathic relations between trees and herbaceous understory vegetation.

Key Words—Phenolic acids, allelopathy, woodland plants, germination, early growth, *Deschampsia flexuosa*, *Scrophularia nodosa*, *Senecio sylvaticus*, *Chamaenerion angustifolium*.

INTRODUCTION

In forestry, litter is recognized as a potential selective agent for germination and growth of tree seedlings (Leibundgut, 1976; Rice, 1984; Becker and Drapier, 1984) and for herbaceous woodland plants (Lohdi, 1975; Ahlgren and

Ahlgren, 1981; Lohdi and Killingbeck, 1982). The toxic effects are often ascribed to phenolic substances (Flaig, 1973; Rice, 1984) released in high amounts from decomposing litter (Anderson, 1973; Blaschke, 1979; Kuiters and Sarink, 1986). Although polymerization to more complex humus compounds such as humic and fulvic acids occurs (Flaig, 1973), phenolic acids are permanently present in the soil solution with some seasonal fluctuation (Lohdi, 1975; Kuiters and Denneman, 1987). Due to their water solubility and their relatively simple molecular structure, they are easily taken up by plants (Flaig, 1973).

Many investigations have revealed that phenolics can seriously interfere with metabolic processes during germination, seedling, and later growth stages. Most of these studies have been carried out with crop or weed species (Demos et al., 1975; Rasmussen and Einhellig, 1977; Einhellig and Rasmussen, 1978; Lynch, 1980; Williams and Hoagland, 1982; Blum et al., 1984; Nandakumar and Rangaswamy, 1985), and little information is available about the sensitivity of woodland plants for phenolic substances. In this study we investigated the effects of commonly occurring phenolic acids on the germination of several woodland (-clearing) species and studied their effects on early seedling growth in more detail.

METHODS AND MATERIALS

Germination and Radicle Elongation

Four plant species, i.e., *Scrophularia nodosa* L., *Deschampsia flexuosa* (L.) Trin., *Senecio sylvaticus* L., and *Chamaenerion angustifolium* (L.) Scop., were tested in separate experiments for their sensitivity to phenolic acids. Seeds were collected at several woodland clearings in The Netherlands during the summer of 1984. Fifty seeds were placed on Whatman No. 1 paper on a plastic floater drifting on 50 ml test solution in small plastic containers (8 × 8 × 10 cm). The test solution consisted of phenolic acids commonly occurring in litter and soil of woodlands (Rice, 1984; Whitehead et al., 1982; Kuiters and Sarink, 1986; Kuiters and Denneman, 1987). The acids *p*-hydroxybenzoic, vanillic, caffeic, salicylic, syringic, *p*-coumaric, and ferulic were each tested in concentrations of 0, 0.01, 0.5, and 10 mM. In addition, an equimolar mixture of the seven phenolic acids, where the total phenolic acid concentration was either 0, 0.01, 0.5, or 10 mM, was tested. Each treatment was replicated five times. The plastic containers were placed at random in a climate room with a light-dark regime of 16:8 hr, light intensity of 50 W/m², temperature 21°C ± 3, and a relative humidity of 70%. The number of germinated seeds was recorded each day for 35 days, although germination was completed after 28 days. A seed was considered germinated if the radicle protruded from the seed coat by at

least 1 mm. Three variables were measured: total seed germination, 50% germination day, and radicle length of the 15 first germinated seedlings after 30 days. The 50% germination day (the day on which 50% of the total germinated seeds had germinated) was used as variable measuring the germination rate. All variables were calculated as percentage of the control (distilled water). For *Scrophularia* no radicle elongation was measured. Data were tested for homogeneity with Bartlett's test and, if necessary, were log-transformed. The statistical model was a two-way analysis of variance (Sokal and Rohlf, 1981). Although no statistical comparisons could be made between plant species, as they were tested in distinct experiments, some general observations could be made.

Early Seedling Growth

In an additional experiment, early seedling growth was studied in more detail. Plastic pots were filled with plastic beads and 300 ml test solution. Two phenolic acids, ferulic and *p*-coumaric, were used in concentrations of 0, 0.01, 0.5, and 1 mM. The phenolic acids were added to a 0.1 strength Hoagland solution (Hoagland and Arnon, 1950). A combined treatment (total concentration: 1 mM) was also used to determine if interference between both compounds occurs. Seven-day-old seedlings of either *Deschampsia* or *Senecio* were placed in the plastic pots. Each treatment was replicated three times, with eight seedlings per pot. After 21 days, plants were harvested and five variables were measured: dry weight, primary root length, number and length of secondary roots, and shoot length. Data were analyzed per plant species using Bartlett's test for homogeneity. If necessary data were log-transformed before a two-way ANOVA model was used (Sokal and Rohlf, 1981).

RESULTS

Germination Experiment

Total Seed Germination. The phenolic acids only slightly reduced total seed germination (Figure 1), although from Table 1 it can be concluded that for all species except *Scrophularia*, the effects were significant ($P < 0.001$). Moreover, the effects were dependent on the phenolic compound ($P < 0.001$). At concentrations of 0.01 and 0.5 mM neither of the phenolic acids influenced total seed germination. Only at the highest concentration (10 mM) was germination inhibited by some of the compounds. Salicylic acid and caffeic acid had the largest and smallest effect on total seed germination, respectively. The complete inhibition of germination of *Chamaenerion* seeds on 10 mM vanillic acid is striking. The other species were less severely inhibited by this compound. In

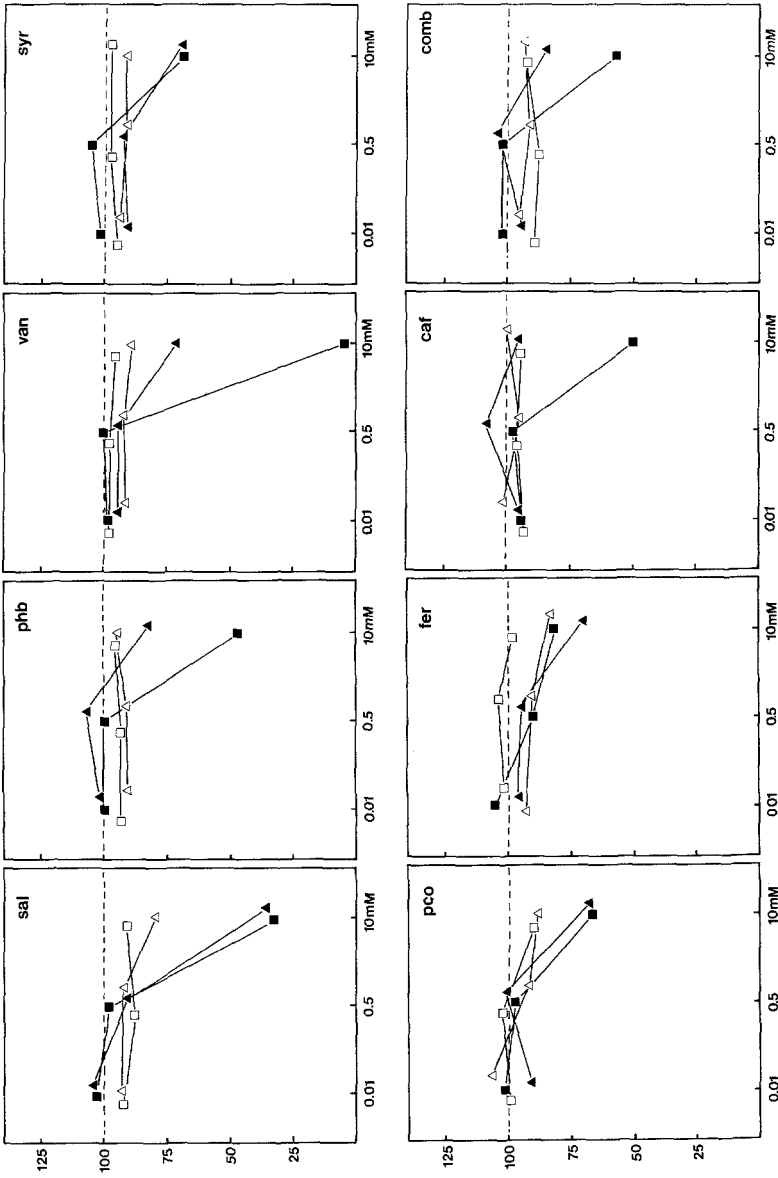


FIG. 1. Effects of phenolic acids on total seed germination of four herbaceous woodland species: ■ *Chamaenerion angustifolium*; □ *Scrophularia nodosa*; ▲ *Deschampsia flexuosa*; △ *Senecio sylvaticus*. sal = salicylic acid; phb = *p*-hydroxybenzoic acid; van = vanillic acid; syr = syringic acid; pco = *p*-coumaric acid; fer = ferulic acid; caf = caffeic acid and comb = an equimolar mixture of these compounds. Control is 100%.

TABLE 1. SIGNIFICANCE LEVELS OF PHENOLIC ACIDS ON TOTAL SEED GERMINATION, 50% GERMINATION DAY, AND RADICLE LENGTH FOR TESTED PLANT SPECIES^a

Source of variation	Plant species											
	<i>Scrophularia nodosa</i>			<i>Deschampsia flexuosa</i>			<i>Senecio sylvaticus</i>			<i>Chamaenerion angustifolium</i>		
	Total seed germination	50% GD		Total seed germination	50% GD	Radicle length	Total seed germination	50% GD	Radicle length	Total seed germination	50% GD	Radicle length
Phenolic acids	ns	***		***	***	***	***	***	***	***	***	***
Type of compound	*	***		***	*	***	*	***	***	***	***	***
Interaction	ns	**		***	*	***	***	***	***	***	***	*

^aSignificance levels: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

general, *Chamaenerion* and *Deschampsia* were most inhibited, whereas *Scrophularia* and *Senecio* were hardly affected by the phenolic acids.

50% Germination Day. The 50% germination day was used as a measure for the germination rate. This variable was slightly more affected by the phenolic compounds than total seed germination (Figure 2). Phenolic acids had a significant effect on the 50% germination day of all plant species ($P < 0.001$), and the effects were dependent on the type of phenolic compound (Table 1). At 0.01 and 0.5 mM, no distinct effects occurred, but at 10 mM the effects were considerable. At a concentration of 10 mM salicylic acid, the germination rate of seeds of *Chamaenerion* and *Deschampsia* was delayed by 260% and 150%, respectively. Germination rate of seeds of *Scrophularia* and *Senecio* was much less influenced.

Radicle Elongation. Radicle elongation was much more affected than germination (Figure 3). Phenolic acids had a significant effect on the radicle-length of the seedlings of each plant species ($P < 0.001$), and these effects were dependent on the phenolic compound (Table 1). At the lower concentrations (0.01 and 0.5 mM) some stimulatory effects occurred, especially by *Senecio*, but most of these roots were threadlike with very few hair roots. Radicle elongation of *Senecio* and *Chamaenerion* was severely inhibited by all phenolic acids at 10 mM. The radicle elongation of seedlings of *Deschampsia* was relatively unaffected by the phenolic compounds.

Order of Effectiveness of Phenolic Compounds. A comparison of the effectiveness of the phenolic acids (averaged over all tested plant species) revealed the following order from high to low:

Total seed germination: salicylic > ferulic > vanillic > syringic = *p*-coumaric > *p*-hydroxybenzoic = caffeic acid.

50% germination: salicylic > vanillic > ferulic = *p*-coumaric > *p*-hydroxybenzoic > syringic > caffeic acid.

Radicle elongation: *p*-coumaric > ferulic > salicylic > *p*-hydroxybenzoic > vanillic = syringic = caffeic acid.

The order differed slightly between the tested parameters. A comparison of the effects of the combination of compounds with each of the phenolic acids indicates that the effectiveness of each compound hardly changed when applied in combination with the others.

Seedling Experiment

Ferulic and *p*-coumaric acid affected seedling development of both *Deschampsia* and *Senecio* (Table 2). The phenolic acids had a significant effect on primary root length ($P < 0.001$), on number ($P < 0.01$) and length of secondary roots ($P < 0.01$), but not on shoot length (Table 3). With increasing phe-

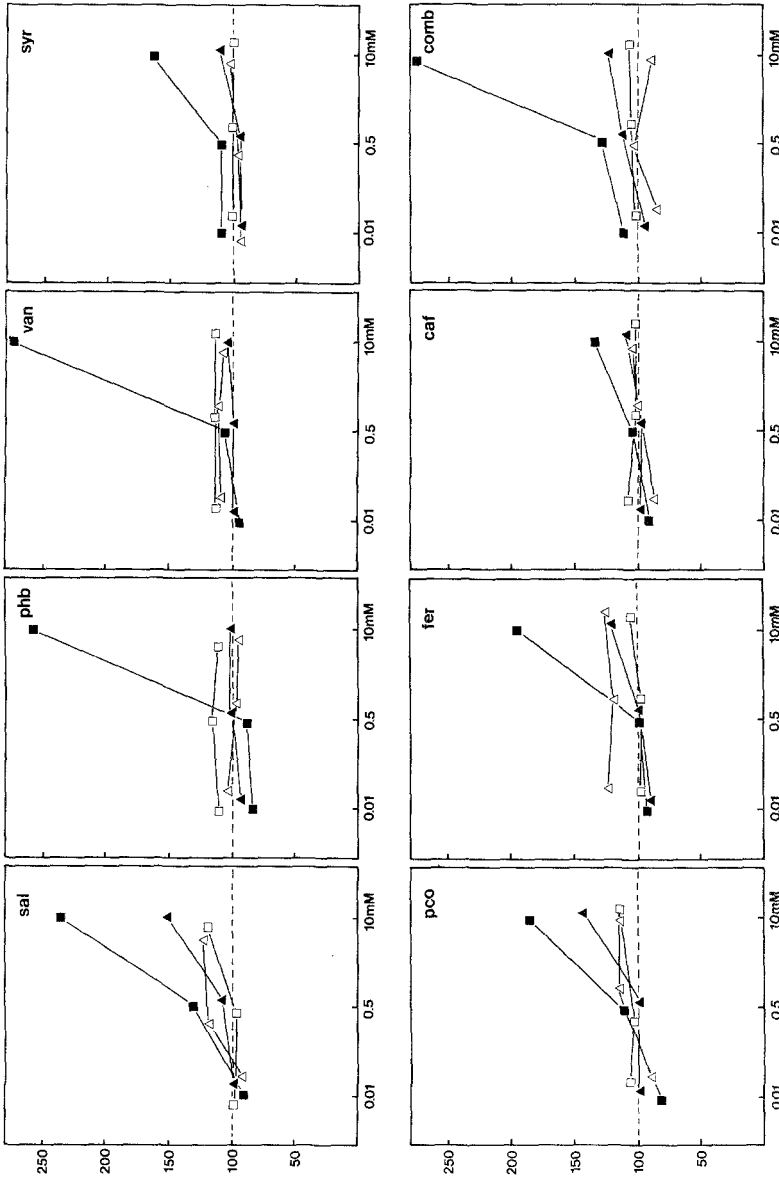


FIG. 2. Effects of phenolic acids on 50% germination day of four herbaceous woodland species: ■ *Chamaenerion angustifolium*; □ *Senecio sylvaticus*; ▲ *Deschampsia flexuosa*; △ *Senecio sylvaticus*. sal = salicylic acid; phb = *p*-hydroxybenzoic acid; van = vanillic acid; syr = syringic acid; pco = *p*-coumaric acid; fer = ferulic acid; caf = caffeic acid and comb = an equimolar mixture of these compounds. Control is 100%.

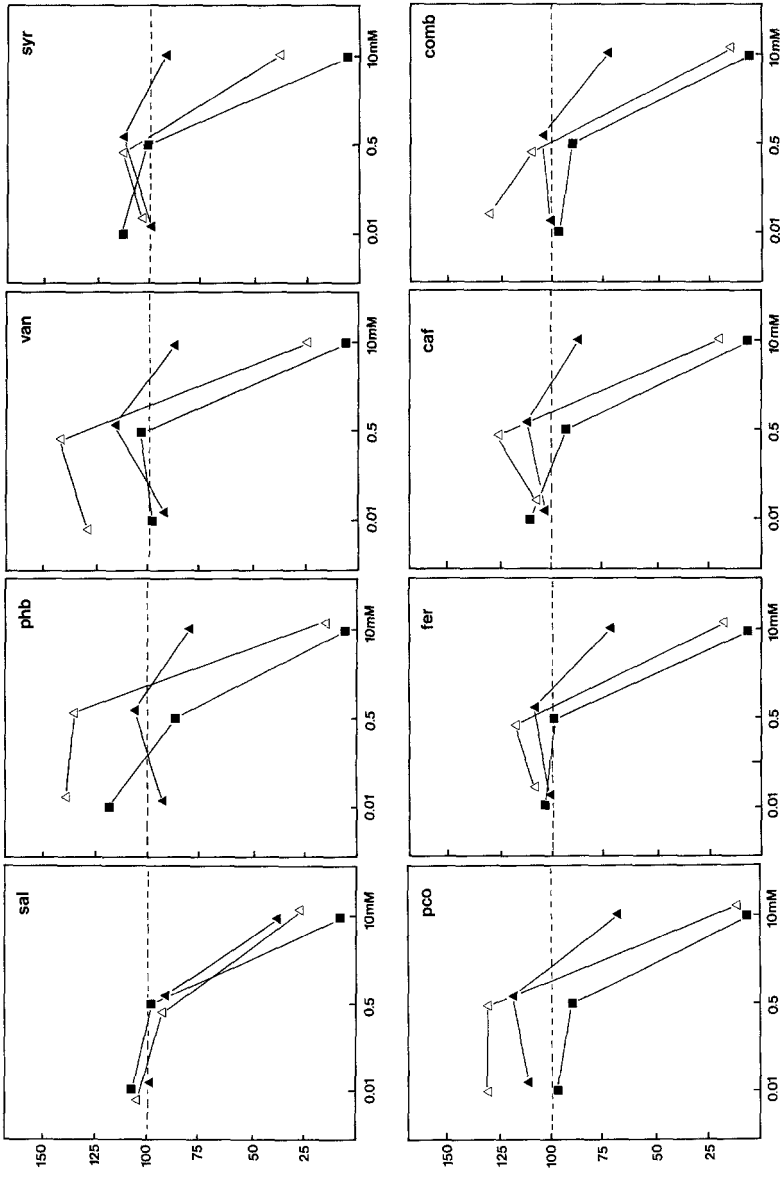


FIG. 3. Effects of phenolic acids on radicle length of three herbaceous woodland species, 35 days after the start of the experiment. ■ *Chamaenerion angustifolium*; ▲ *Deschampsia flexuosa*; △ *Senecio sylvaticus*. sal = salicylic acid; phb = *p*-hydroxybenzoic acid; van = vanillic acid; syr = syringic acid; pco = *p*-coumaric acid; fer = ferulic acid; caf = caffeic acid and comb = an equimolar mixture of these compounds. Control is 100%.

TABLE 2. EFFECTS OF FERULIC AND *p*-COUMARIC ACIDS ON SEVERAL PARAMETERS (Means \pm Standard Deviation) OF EARLY SEEDLING GROWTH OF *Senecio sylvaticus* AND *Deschampsia flexuosa*

Treatment	Dry weight (μg)	Primary root length (mm)	Secondary root length (mm)	Number	Shoot length (mm)
<i>Senecio sylvaticus</i>					
Control	685 \pm 322	38 \pm 39	14 \pm 10	3.2 \pm 0.7	2.8 \pm 1.1
Ferulic acid					
0.01 mM	974 \pm 69	20 \pm 2	13 \pm 1	2.4 \pm 0.5	3.0 \pm 0.6
0.5	500 \pm 154	22 \pm 7	7 \pm 11	1.8 \pm 2.0	2.5 \pm 1.0
1.0	579 \pm 85	22 \pm 3	9 \pm 5	2.1 \pm 0.7	4.0 \pm 1.4
<i>p</i> -Coumaric acid					
0.01 mM	823 \pm 159	27 \pm 4	34 \pm 12	4.0 \pm 0.5	4.7 \pm 2.5
0.5	742 \pm 297	25 \pm 12	16 \pm 17	2.4 \pm 1.2	3.3 \pm 0.8
1.0	551 \pm 67	19 \pm 3	10 \pm 6	2.1 \pm 0.5	3.2 \pm 0.7
Combination					
ferulic acid (0.5 mM) <i>p</i> -coumaric acid (0.5 mM)	677 \pm 190	26 \pm 5	13 \pm 10	2.4 \pm 0.8	3.8 \pm 0.6
<i>Deschampsia flexuosa</i>					
Control	979 \pm 230	33 \pm 1	26 \pm 1	7.2 \pm 0.9	37 \pm 2
Ferulic acid					
0.01 mM	982 \pm 304	37 \pm 3	34 \pm 23	8.1 \pm 1.8	39 \pm 2
0.5	1081 \pm 26	35 \pm 6	25 \pm 8	8.1 \pm 1.4	42 \pm 7
1.0	823 \pm 131	27 \pm 2	20 \pm 4	5.1 \pm 1.0	32 \pm 4
<i>p</i> -Coumaric acid					
0.01 mM	1161 \pm 223	57 \pm 2	53 \pm 32	10.0 \pm 0.7	50 \pm 8
0.5	1038 \pm 264	37 \pm 10	23 \pm 6	7.2 \pm 1.5	37 \pm 2
1.0	1031 \pm 294	28 \pm 7	27 \pm 9	5.4 \pm 1.1	37 \pm 4
Combination					
ferulic acid (0.5 mM) <i>p</i> -coumaric acid (0.5 mM)	1000 \pm 358	31 \pm 1	24 \pm 5	5.3 \pm 1.6	39 \pm 4

nolic acid concentration, the dry weight of the seedlings decreased, although this effect was not significant. Effects were strongly dependent on the plant species ($P < 0.001$). No significant differences could be established between these phenolic compounds. The effects were most pronounced for *Deschampsia*, in which primary root length and secondary root development was largely stimulated at 0.01 mM *p*-coumaric acid. At the intermediate concentration of 0.5 mM of both compounds, no distinct effects were observed. At the highest concentration of both compounds (1 mM) the number of secondary roots was reduced, whereas the other parameters were hardly influenced. Comparing the effects of the combined treatment (0.5 mM of each compound) with those of

TABLE 3. LEVELS OF SIGNIFICANCE OF EFFECTS OF FERULIC AND *p*-COUMARIC ACID TREATMENTS ON DIFFERENT GROWTH PARAMETERS OF *Senecio sylvaticus* AND *Deschampsia flexuosa*^a

Source of variation	Variable				
	Dry weight length	Primary root length	Secondary roots		Shoot lengths
			Length	Number	
Phenolic acids	ns	***	**	**	ns
Type of compound	ns	ns	ns	ns	ns
Plant species	***	***	***	***	***
Phen. × type	ns	ns	ns	ns	ns
Phen. × plant sp.	ns	*	ns	ns	ns
Type × plant sp.	ns	ns	ns	ns	ns
Phen. × type × plant sp.	ns	ns	ns	ns	ns

^a* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant.

the treatments where the phenolic acids were added singly in concentrations of 0.5 or 1 mM, reveals that the effects were related to the total concentration rather than to an additional or synergistic effect of each individual compound.

DISCUSSION

Both experiments clearly indicate that phenolic compounds occurring in organic-rich soils can influence germination and seedling development of herbaceous plant species. The observed effects are highly dependent on the phenolic acid concentrations. Low concentrations of 0.01 mM often have a stimulatory effect, whereas concentrations of 1 mM and higher have an inhibitory effect. This was also observed by Scheffer and Ulrich (1960) and Flaig (1973). Germination was delayed rather than inhibited, conforming to the results of Williams and Hoagland (1982). Under field conditions, where competitive interference between herbs occurs, delay of seed germination may be as important as the inhibition of germination (Van Baalen et al., 1984). Root development was severely affected by phenolic compounds, as also found in other investigations (Hennequin and Juste, 1967; Rasmussen and Einhellig, 1977; Williams and Hoagland, 1982). Besides primary root elongation, the development and growth of secondary roots was severely affected. Low concentrations of *p*-coumaric and ferulic acid stimulated the development of secondary roots. Large differences in effectiveness were observed between the tested phenolic

acids. Salicylic, *p*-coumaric, and ferulic acids were most effective, while the effects of syringic and caffeic acids were relatively small. The order of activity we found more or less resembled that of Reynolds (1978), who used lettuce as test species. Apparently, the effectiveness of phenolic acids is determined to a large extent by chemical structure, but the sensitivity of the tested plant species clearly varied. No evidence was found in these experiments showing that the effects of phenolic acids on germination or radicle development are different when the compounds are applied in combination.

Several mechanisms for the effect of phenolic acids on germination and development of plants have been suggested. Phenolic acids may interfere with IAA (indole-acetic acid) metabolism, mitochondrial metabolism and respiration, photosynthesis, synthesis of proteins, and ion uptake and transport (Rice, 1984; Einhellig, 1986).

Among the tested species, *Deschampsia flexuosa* was the least and *Chamaenerion angustifolium* the most affected species. Compared to crop or weed species, the four species we tested were less sensitive to phenolic acids. Growth inhibition occurred beyond 1 mM, whereas crop species are often inhibited above a threshold value of 0.25 mM (Williams and Hoagland, 1982; Rasmussen and Einhellig, 1977; Einhellig and Rasmussen, 1978). The large amounts of phenolics present in forest leaf litter may have resulted in a selection so that woodland herbs are better adapted to these organic compounds than crop species.

Nevertheless, even this resistance may not be sufficient, as demonstrated by the inhibition of seedling development by high concentrations of phenolic acids. The high concentrations of phenolic substances in autumn during and after the leaf fall period (Kuiters and Sarink, 1986) may act as such a severe selecting agent (combined with mechanical stress) that germination in spring is favored. Indeed, most woodland herbs are spring germinators (Grime et al., 1981). The selection of a winter annual type of *Senecio sylvaticus* in Dutch coastal forests, however, may indicate that the factors determining germination time may be more complex (Ernst, 1985). The seedling stage seems to be a sensitive phase in the life cycle of plants with respect to phenolic compounds in the humus layers. The success of establishment of emerging seedlings is, besides other factors, presumably largely influenced by the chemical impacts of litter. The species composition of the herb layer in forests may be partly dependent on the litter type and not just on the preference of species for certain abiotic conditions, like shade, water, or nutrient conditions. Further knowledge of the effects of forest litter on germination and subsequent growth of seedlings, especially in relation to other soil factors (Ernst and Nelissen, 1979), is essential for a better understanding of species distribution and succession phenomena in forests.

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CHARACTERIZATION AND PARTIAL PURIFICATION OF ATTRACTANTS FOR NEMATODE *Orrina phyllobia* FROM FOLIAGE OF *Solanum elaeagnifolium*

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Abstract—An unknown attractant for the nematode *Orrina phyllobia* was extracted with water from foliage of *Solanum elaeagnifolium*. Stability, solubility, ionic character, and chromatographic purification were investigated using a bioassay based on nematode aggregation in agar. Activity was non-volatile, dialyzable, heat stable below 60°C, and partially lost within 30 min at 100°C. Activity was unchanged from pH 5 to 12, but was entirely lost at pH 2. Loss of activity at low pH did not appear to result from direct effects of pH on nematode behavior and was partially recovered by readjustment to pH 7. The attractive factor was most soluble in water and appeared to be cationically but not anionically exchangeable. Activity appeared to chromatograph as several compounds by high-performance liquid chromatography employing reverse phase C₁₈ and amine-bonded columns. Various known compounds that are common to *Solanum* spp. or that attract other nematodes were unattractive. Extraction of *S. elaeagnifolium* foliage specifically for solanaceous glycoalkaloids using methods developed for *S. tuberosum* did not yield an attractive product.

Key Words—*Orrina phyllobia*, *Solanum elaeagnifolium*, foliar nematode, host finding, nematode chemotaxis, silverleaf nightshade.

INTRODUCTION

More than 4000 nematode species parasitize plants and considerable research has been done toward learning how soil-borne infective stages find roots and stems (reviewed by Steiner, 1925; Croll, 1970; Green, 1971; Prot, 1980; Zuckerman and Jansson, 1984). Nematode orientation to temperature and soil mois-

ture gradients may assist host finding, but most evidence indicates chemotaxis is primarily responsible. Very little is known concerning specifically phytogetic attractants. Many nematodes are attracted to root leachates; in at least two cases, root leachate attractiveness was removed with activated charcoal, suggesting an organic nature (Miller and McIntyre, 1976; Peacock, 1961). Various common biogenic chemicals, largely amino acids, attract or repel some bacteriophagous, phytoparasitic, and zooparasitic nematodes on agar or agarose gels (Dusenbery, 1983; Pye and Burman, 1981; J.B. Ward et al., 1984; S. Ward, 1973). These include L-tryptophan, glutamic acid, aspartic acid, cysteine, L-tyrosine and other amines, cAMP, cGMP, gibberellic acid, glutathione, ascorbic acid, and pyridine. These responses were discovered by screening hundreds of known compounds and none has been established to mediate orientation within soil (Dusenbery, 1983). Failure to identify plant-specific attractants has encouraged the view (Klingler, 1965; Prot, 1980; Wallace, 1973) that phytoparasitic nematodes in soil may rely primarily on nonspecific chemical stimuli, such as CO₂ and inorganic ions for orientation. Several species orient on gradients of CO₂, Na⁺, Cl⁻, or OH⁻.

Progress toward understanding nematode chemotaxis as it relates to specific plant hosts has been made with the pine-wood nematode, *Bursaphelenchus xylophilus* (Futai, 1980). Various terpenes and other compounds common to the coniferous plants are attractive or repellant in vitro (Tominaga et al., 1983, 1984). The life cycle of this nematode differs from most phytoparasitic species in that it is normally transmitted to the trunk and branches of the host tree by a pine sawyer beetle (*Monochamus alternatus*), rather than by moving through soil. Thereafter, nematodes move and feed within resin canals where behavioral responses to terpenes may play an important role. Chemotactic preferences for tree saps appear related to host suitability among tree genera but not among pine species (Futai, 1980).

A second nematode whose behavior appears attuned to specific plants is *Orrina phyllobia*. It is known to parasitize only foliar parts of certain plants within the genus *Solanum* and is specifically attracted to leachates from stems of four *Solanum* spp. These are *S. tuberosum* (potato), *S. melongena* (eggplant), *S. carolinense* (Carolina horse nettle), and *S. elaeagnifolium* (silverleaf nightshade). Leachates from rhizomes of *Solanum* spp. and from stems of 30 plant species in other genera spanning 12 plant families were not attractive (Robinson et al., 1979). Attraction to stems is ecologically valuable to *O. phyllobia* because its life cycle entails aggregation of infective juveniles, which are about 700 μm long, within soil around stem bases and emerging shoot tips. When stem surfaces are moist, the juveniles ascend as far as 50 cm and invade foliar meristems to initiate new infections (Robinson et al., 1978). We have begun to characterize and chromatographically purify attractant from foliage of the common host,

S. elaeagnifolium, with a view toward understanding this rather host-specific behavior by a phytoparasitic nematode.

METHODS AND MATERIALS

Bioassay Procedure. Stems and leaves of *S. elaeagnifolium* were collected from field-grown plants, freeze dried intact, and ground with a Wiley mill. The powder obtained was stored in a desiccator over anhydrous CaSO_4 and used as a standard source of attractant. Preparations from plants collected on other occasions were similarly attractive. The assay we adopted was a modification of methods described by Balan et al. (1976) and Tominaga et al. (1983) for studying chemotaxis in other nematodes. Infective juveniles of *O. phyllobia* were removed daily from dried leaf galls of *S. elaeagnifolium* as previously described (Robinson et al., 1979) and suspended in a continuously aerated dilute salt solution (4.5 mM NaCl, 0.4 mM KCl, 0.05 mM CaCl_2 , 0.05 mM MgCl_2) to reduce ion regulation stress. Bacto-Agar (1.5%, Difco) was dissolved in water (w/w) supplemented with dilute salts equivalent to those in the nematode suspension. Equal parts of nematode suspension (22°C) and agar (42°C) were then mixed and poured into a 35-mm-diameter Petri dish to obtain a 2-mm-deep film of 0.75% agar containing about 3000 juveniles. The mixing procedure induced a brief reduction in motility from which nematodes completely recovered within several minutes. A 3-mm-diameter disk of filter paper (Whatman No. 1) saturated with the test solution and air-dried was placed halfway between the center and the edge of the dish. Trials with aqueous solutions of the biological stain safranin-O indicated that the pattern of diffusion of material from the dried disk was hemispherical downward. Two untreated disks were then placed equidistant from the center, 1 cm from each side of the treated disk. After 90 min, a 5-mm cork borer and small spatula were used to transfer agar cylinders containing the disks to counting dishes where the nematodes within each cylinder were dispersed into water and microscopically counted.

The response index (*RI*) was defined as $RI = T/(T + U)$ where *T* = nematodes under the treated disk and *U* = average number of nematodes under untreated disks in the same dish. *RI* > 50% indicated aggregation and *RI* < 50%, repulsion. *RI* values were binomially distributed and examined statistically after arcsine transformation. In each experiment except where noted, five dilutions spanning two orders of magnitude were prepared for bioassay from filtered aqueous extract of the dry plant powder (6% w/w). The 6% concentration will be referred to as standard foliar extract (SFE).

Nematode chemotaxis experiments are frequently conducted without light to mimic soil conditions. In preliminary experiments, we found that bioassay

results obtained under laboratory light were identical to results obtained in the dark. Small temperature gradients (ca. $0.1^{\circ}\text{C}/\text{cm}$) that resulted from differential evaporation of water within dishes strongly influenced nematode distributions. However, dishes were covered and oriented identically to minimize the effect. We recognized that test solutions might generate behaviorally important extremes of pH, ionic strength, and osmotic potential. To evaluate their contributions, we bioassayed aqueous solutions of acetic acid, HCl, and NaOH at 0.01, 0.1, 1, 10, and 100 mM. We also bioassayed solutions with osmotic pressures from 100 to 500 mM by supplementing water and foliar extract with sucrose or inorganic ions in ratios described. Standard foliar extract had 100–120 mM osmotic pressure by freezing point depression and pH 6.3–6.5. Bioassays were performed in five replicates. Experiments were repeated at least once. Reproducibility was sometimes confirmed by assigning numerical ratings to aggregations instead of counting nematodes.

Preliminary Characterization of Attractants. Standard foliar extract was bioassayed after each of the following procedures: dialysis against distilled water across 3500 MWCO dialysis tubing; filtration through activated charcoal; heating to 50, 60, 70, 90, and 100°C for various intervals up to 75 min; pH adjustment with HCl/NaOH; and readjustment to pH 7. Extract had appreciable buffering capacity near pH 6.5, and neutralization beyond 3 pH units required excessive dilution of extract or increases in Na^{+} and Cl^{-} concentrations >200 mM. These are physiologically significant levels; therefore, control solutions were prepared with similar osmotic pressures by adding NaCl to extract without pH adjustment. To examine attractant solubility, foliar powder was extracted (2% w/w) with solvent mixtures in a polarity series including water, methanol, 95% ethanol, 1-propanol, 1-butanol, diethyl ether, and hexane. Each extract was then filtered, evaporated to dryness, and reextracted with water at original volume. Residue obtained from the first filtration was separately reextracted with water and all solutions assayed. Ionic character was examined with pre-filled Poly-Prep columns (Bio-Rad) containing 2 ml of AG 50W-X8 and AG 1-X8 ion exchange resins in the H^{+} and Cl^{-} forms. Aqueous extract was prepared at 67% the concentration of SFE. After washing with distilled water, resin was drained under vacuum, and 3 ml of extract was passed through each column. Columns were then washed with 9 ml distilled water and eluted with 4 ml 100 mM KNO_3 , followed by another 4 ml 1 M KNO_3 . Eluates and washes were bioassayed before and after adjustment to pH 7 with NaOH.

Toward HPLC Purification. Preliminary separations were made with a Waters HPLC system equipped with a reverse phase C_{18} Semi-Prep column (7.8 mm \times 30 cm) (Waters) and a variable wavelength detector set at 254 nm. Solvent flow was 1.5 ml/min as follows: 15 min isocratic water, 10 min linear gradient to 100% methanol, 10 min hold, 20 min linear gradient to 100% water. Eleven 5-min fractions were collected separately from three 50- μl injections of

67% SFE, composited, evaporated to dryness (60°C), redissolved with 2 ml water, then reevaporated to dryness in small dishes under vacuum (50°C), and finally redissolved in 150 μ l water for bioassay. Solvent controls were also tested. A cleanup procedure using disposable C₁₈ preparatory columns (Sep Pak, Waters) was then developed based on the column volume equivalents of eluants needed for a stepwise approximation to the HPLC solvent gradient. Active fractions were prepared in quantity with the cleanup procedure. Further separations were done with an analytical C₁₈ Radial Pak cartridge (8 mm \times 10 cm) or an amine-bonded column (3.9 mm \times 30 cm) (Waters). Mobile phase for the C₁₈ cartridge was a 25-min water-methanol linear gradient (1.0 ml/min) from 20% to 80% methanol. Mobile phase for the amine-bonded column was a 20-min CH₃CN-water linear gradient (2.0 ml/min) from 90% to 65% CH₃CN, a 5-min hold, and a linear gradient back to initial conditions. Thirty-five 1-min fractions were collected separately from two injections, composited, evaporated, reextracted with 25 μ l water, and bioassayed.

Examination of Known Substances for Activity. Commercial preparations (Sigma) of various compounds were bioassayed because they are associated with *Solanum* spp. or because they have chemotactic activity to other nematodes. These included the common solanaceous glycoalkaloids α -chaconine and α -solanine at pH 7.5. With the view that attractants might occur among other *Solanum*-specific glycoalkaloids, the procedures described by Allen and Kuc (1968), by Bushway et al. (1985), and by Carman et al. (1986) for extracting glycoalkaloids from potato were applied to the *S. elaeagnifolium* foliar powder and confirmed by HPLC of product against commercial preparations of α -chaconine and α -solanine.

RESULTS AND DISCUSSION

Bioassay Procedure. The *RI* reached equilibrium after ca. 60 min (Figure 1A). The *RI* always approximated a log decay function of concentration that asymptotically approached 85–95%. The least-squares line relating *RI* and log₁₀ concentration was employed to express bioassay results in terms of the apparent concentration of attractant as well as in *RI* units (Figure 1B). Results of one experiment (Figure 3 below) are presented in both ways to convey the exponential relationship between attractant concentration and statistical uncertainty. Independent effects of pH on the bioassay were not detected with acetic acid and HCl solutions of pH > 2. NaOH > 10 mM caused weak aggregation and at pH 12 was equivalent to the effect of 3% SFE. Adding sucrose or mixed salts to water and foliar extract to generate osmotic pressures as high as 500 mM did not change bioassay results. Osmotic pressures of test solutions in subsequent experiments were <300 mM. Some species of nematodes respond

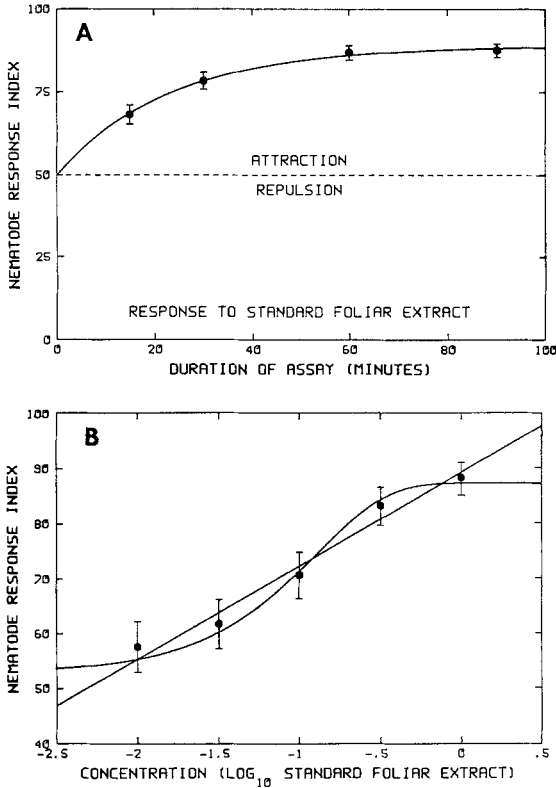


FIG. 1. Effects of bioassay duration and foliar attractant concentration on the nematode response index. (A) Increase in nematode response during 90-min exposure to aqueous extract of *Solanum elaeagnifolium* foliage. (B) Representative concentration-response curve for aqueous foliar extract. Concentrations are expressed as percentage of a standard concentration of dry foliage in water (6% w/w). Brackets indicate confidence intervals for five replicates obtained after arcsine transformation. The straight line and the sigmoid log decay function [$dy/dx = (Y - y)K$, where y = response index, x = concentration, Y = the maximum value of y , and K = a constant] were fitted by least squares.

behaviorally to pH, ion, and osmotic pressure gradients much smaller than we imposed (Dusenbery, 1983; Prot, 1980). If *O. phyllobia* also responds to them, their effects are very small under our assay conditions compared with the effects of attractant within *S. elaeagnifolium* foliage.

Preliminary Characterization of Attractants. Attractant from *S. elaeagnifolium* was essentially nonvolatile, freely dialyzable, and heat stable for 90 min under the conditions we used for evaporating test solutions ($< 60^{\circ}\text{C}$). How-

ever, some activity was lost within 30 min at 100°C (Figure 2A). Further losses were not detected up to 90 min, suggesting that heating generated a repulsive product or that two or more attractants were present and differed in temperature sensitivity. Activity was highly polar or ionic in character, not removed with activated charcoal, and most soluble in water (Figure 3). The attractiveness of standard foliar extract was completely lost by adjusting pH to 2 with HCl but was partially recovered after readjustment to 7 (Figure 2B). Addition of NaCl to standard foliar extract at concentrations equal to those generated by pH adjustment did not reduce activity, suggesting that direct effects of Na^+ and Cl^- on nematode behavior were not important factors. Disks with SFE at low pH frequently induced nematodes to aggregate in a halolike ring surrounding the disk 2–4 mm from its edge. When a second disk, which had been saturated with 0.1 N NaOH and dried, was placed on top of the first disk, the nematodes

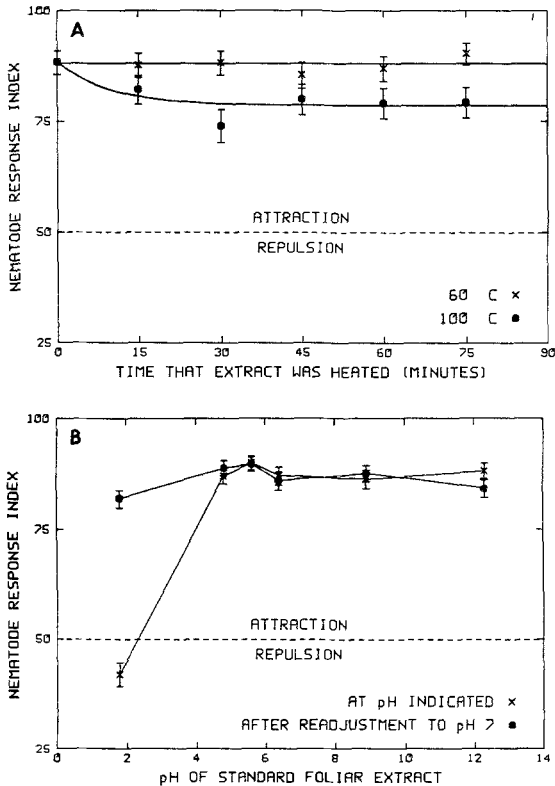


FIG. 2. Stability of nematode attractiveness of aqueous extract of *Solanum elaeagnifolium* foliage. (A) Partial loss of activity at 100°C. (B) Partially irreversible loss of activity at low pH. Brackets indicate confidence intervals as in Figure 1.

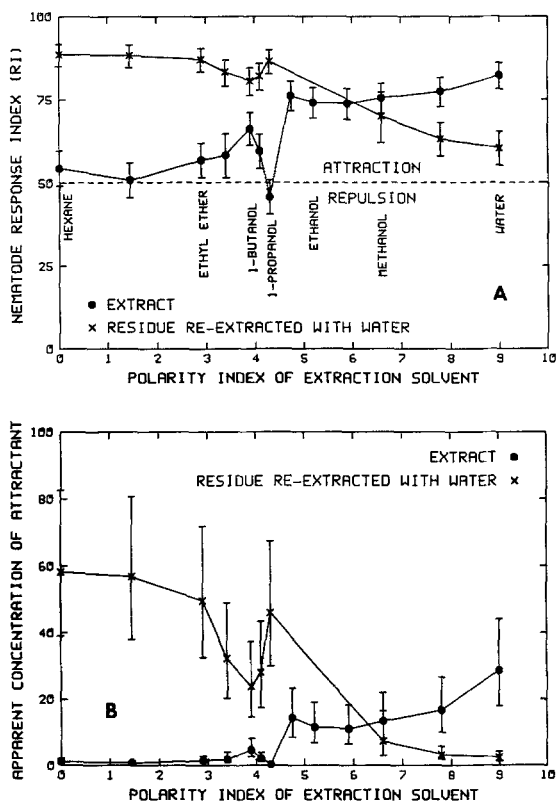


FIG. 3. (A) Effect of solvent polarity on extraction of attractants from dry *Solanum elaeagnifolium* foliage (2% w/w). Each extract was filtered, evaporated to dryness, and reextracted with the original volume of water before bioassay. Residue from each filtration also was reextracted with the original volume of water. Brackets indicate confidence intervals as in Fig. 1. (B) Same information as in (A) expressed in terms of the percentage concentration of attractant in a dilution series of a standard aqueous extract (6% w/w) that was bioassayed concurrently. Note the exponential effect of attractant concentration on statistical uncertainty.

comprising the ring accumulated underneath the disks within 15 min. Measurements of agar pH various distances from the disk with a 2-mm micro-pH electrode indicated that pH elevation to about 4 was required for aggregation. This effect may result from repellents or attractants that are pH reversible in activity.

Passage of 67% SFE through anion (Cl^-) and cation (H^+) exchange columns lowered pH to ca. 2 and removed activity. Upon readjustment to pH 7, extract from the anion exchange column was strongly attractive and extract from the cation exchange column was unattractive (Table 1). Elution with 100 mM

TABLE 1. NEMATODE ATTRACTANT IN ELUATES FROM POLY-PREP COLUMNS CONTAINING ION EXCHANGE RESINS AFTER LOADING WITH 3-ml AQUEOUS EXTRACT PREPARED FROM *Solanum elaeagnifolium* FOLIAGE

Eluate	Eluant	Eluant volume (ml)	Anion exchange resin (Cl ⁻ , AG 1-X8)		Cation Exchange resin (H ⁺ , AG 50W-X8)	
			Nematode response index (%) ^a	Apparent attractant concentration (%) ^b	Nematode response index (%)	Apparent attractant concentration (%)
1	Distilled water wash to condition column	4	38-48	0	43-57	1
2	67% standard foliar extract (sample)	3	69-81	50	52-65	3
3	Distilled water wash	9	46-60	1	47-60	1
4	100 mM KNO ₃	4	48-62	2	55-68	5
5	1 M KNO ₃	4	27-41	0	29-42	0

^a Confidence limits ($P = 0.05$) of arcsine-transformed data, expressed as nematode response index as defined in text.

^b Apparent concentration of attractant relative to standard foliar extract (6% w/w).

KNO₃ followed by adjustment to pH 7 yielded weak activity from the cation but not from the anion exchange resin. Therefore, attractant appeared completely unretained by anion exchange. Failure to recover full activity in the 100 mM KNO₃ cation exchange eluate may have resulted from incomplete elution, acid hydrolysis of attractants, or assay interference from NO₃⁻. Both 1 M KNO₃ eluates were repellent.

Toward HPLC Purification. High levels of activity were detected in the 6- to 10- (I) and 31- to 35- (II) min fractions obtained with the C₁₈ Semi-Prep column. Similar activity occurred in the corresponding fractions of the Sep-Pak cleanup (Figure 4). Bioassay results for subfractions of II from the analytical C₁₈ cartridge showed activity in a baseline separated peak eluting at 9.0 min. Fraction I was evaporated to dryness and reextracted with original volumes of CH₃CN-water mixtures. These were bioassayed, and the solubility of activity (Figure 5) was used to calculate appropriate concentrations of subfractions obtained for bioassay from the amine-bonded column. Most activity occurred in three distinct fractions (Figure 6), two of which contained consistently identifiable absorbance peaks at 230 and 254 nm. Absorbance maxima of active fractions occurred at 203 nm, where solvent interference prevented HPLC detection. The first strongly active fraction to elute induced a mean nematode response equivalent to 34% SFE. The material injected was 100 μl of I in 90:10 CH₃CN in water with an activity concentration equivalent to ca. 12% SFE (Figure 5), and fractions were assayed at a final volume of 25 μl. Therefore, the first active fraction to elute accounted for most of the activity injected.

Known Compounds. Several compounds were weakly attractive, but even the strongest response, to α-chaconine, was weaker than to 5% SFE. Pyridine was strongly repellent (Table 2). Pyridine attracts the bacteriophagous nematode *Caenorhabditis elegans* (Dusenbery, 1976). Extraction of the foliar powder specifically for solanaceous glycoalkaloids, using methods established for potato, yielded products that were neither attractive nor repellent at neutral pH.

Our results indicate that several substances within *S. elaeagnifolium* foliar extract attract infective juveniles of *O. phyllobia* in vitro. They appear to be similarly nonvolatile, dialyzable, polar, ionizable, water soluble, sensitive to very low pH, and stable at biological temperatures. We do not know if common functional groups are involved. Small, stable, water-soluble compounds from foliage would seem ideal for attracting nematodes within soil to plant stem bases. Nematodes, in general, move in water films, and optimum physical conditions for locomotion in soil occur at high water contents, near the inflection point of the soil moisture release curve (Wallace, 1968). We cannot be certain that our attractive HPLC fractions contain compounds that naturally occur in behaviorally active concentrations near living plants; we know nothing concerning their anatomical compartmentalization and occurrence in natural foliar leachates. Our approach has been to develop chromatographic methods for

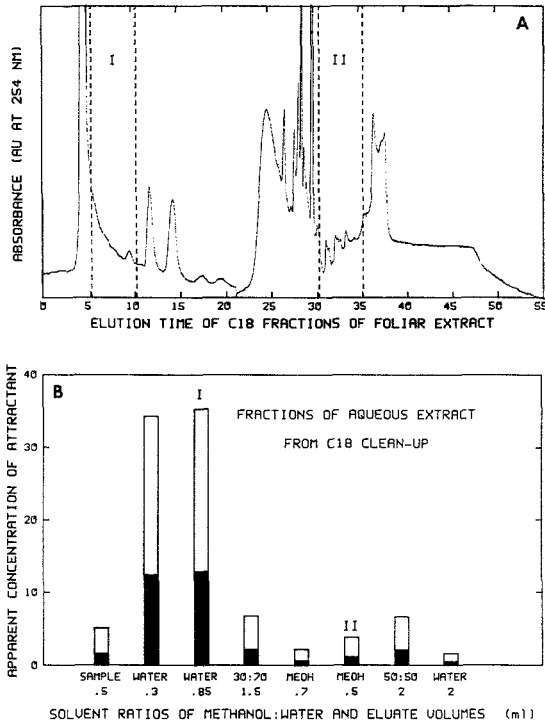


FIG. 4. Preliminary HPLC separation of strong (I) and weak (II) sources of activity in aqueous extract of *Solanum elaeagnifolium* foliage using a water-to-methanol gradient on C₁₈ Semi-Prep column, and assay results for eluates obtained through a C₁₈ Sep Pak cleanup procedure using solvent volumes that approximated the HPLC gradients. (A) UV absorbance for HPLC and locations of activity among eleven 5-min fractions. Solvent concentrations described in text. (B) Nematode response to fractions comparable to the HPLC fractions but obtained with the cleanup procedure. The tops of black and white bars for each fraction indicate the confidence limits ($P = 0.05$) of nematode response expressed as the equivalent percentage concentration of an extract dilution series assayed concurrently as described in Figure 3B.

detecting attractive substances before trying to find them in soil. Further research toward that goal is underway.

The potato cyst nematode, *Globodera rostochiensis*, is a serious agronomic pest in potato production. It is ecologically similar to *O. phyllobia* as a nematode closely associated with *Solanum* spp. hosts. Since 1922, considerable effort has been put toward isolation and identification of a factor in root leachates from *Solanum* spp. that stimulates *G. rostochiensis* egg hatching. The active factor, which is still unidentified, chromatographs as several compounds that

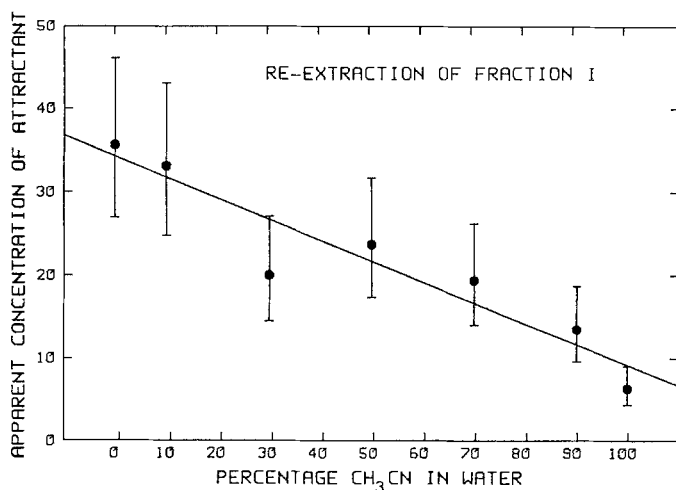


FIG. 5. The apparent concentration of attractant, relative to standard aqueous extract of dry *Solanum elaeagnifolium* foliage (6% w/w), in CH₃CN–water extracts of dry fraction I. Fraction I was obtained by a C₁₈ Sep Pak cleanup procedure, then evaporated to dryness and reextracted with an original volume of the solvent mixture indicated. Note that the concentration of attractant in 90% CH₃CN is only 30% of the concentration in water. Brackets indicate confidence intervals as in Figure 3B.

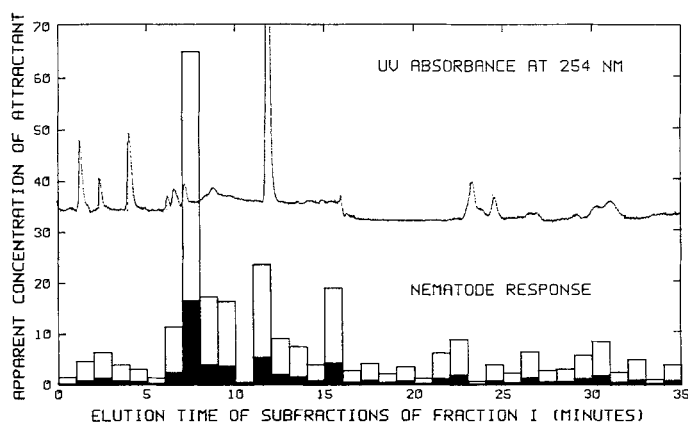


FIG. 6. Nematode attractant activity in 35 1-min HPLC subfractions of fraction I obtained with amine-bonded column and a CH₃CN–water mobile-phase gradient. Solvent concentrations described in text. Bars indicate confidence intervals as in Figure 4B. The strongly attractive fraction eluting at 7–8 min accounted for 75% of the total activity in the injected material.

TABLE 2. KNOWN COMPOUNDS TESTED FOR EFFECTS ON BEHAVIOR OF *Orrina phyllobia*

Compound	Concentration (mM)	pH	Nematode response index confidence limits (%) ^a	Apparent concentration of attractant (% of SFE) ^b
cAMP	20	7.2	49-63	1
L-Tryptophan	20	5.8	51-66	2
D-Tryptophan	20	6.0	56-70	3
Pyridine	150	3.2	16-27 ^d	0
α -Chaconine	^c	7.7	59-73	5
α -Solanine	^c	7.4	47-62	1
Gitoxin	^c	6.2	44-59	1
Saponin	30	6.1	57-71	3
Digitonin	10	5.8	40-55	0
NaCl	25	6.3	53-68	2
NaOH	25	11.5	53-68	2
KCl	25	5.9	58-72	4
KOH	25	11.9	51-65	1
SFE				
100%	98 ^e	6.3	85-91	87
33%	34 ^e	6.3	80-87	44
10%	11 ^e	6.3	66-75	8
3%	5 ^e	6.3	57-66	2
1%	1 ^e	6.3	53-62	1
Distilled water			46-55	0

^a Confidence limits ($P = 0.05$) calculated from arcsine-transformed data for five replicates, then reconverted to nematode response index units as defined in text.

^b Obtained by comparing the average nematode response index for five replicates to a response curve obtained concurrently for five dilutions of SFE spanning two orders of magnitude.

SFE = standard foliar extract (6% w/w, aqueous).

^c Saturated solutions <4 mM by freezing point depression.

^d Significantly repellent at $P = 0.01$.

^e Osmotic pressure (mM) by freezing point depression.

are highly polar and water soluble in character (Atkinson et al., 1987). Before initiating characterization of the *O. phyllobia* attractants, we speculated that they might be solanaceous glycoalkaloids or might be related to the potato cyst nematode hatching factor. Our observations support neither prediction. Saturated solutions of the common *Solanum* glycoalkaloids α -chaconine and α -solanine did not attract *O. phyllobia* appreciably; extraction specifically for glycoalkaloids did not yield attractive products; and attraction to SFE occurred at alkaline pHs (>10), at which many *Solanum* glycoalkaloids precipitate from

water. Recent work on potato cyst nematode hatching factor has shown it to be anionically but not cationically exchangeable (Atkinson et al., 1987); the converse was observed for *O. phyllobia* attractant.

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SEX PHEROMONE COMPONENTS FROM ASIAN CORN
BORER, *Ostrinia furnacalis* (Hubner) (Lepidoptera:
Pyralidae) IN TAIWAN

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Abstract—The female sex pheromone of the Asian corn borer, *Ostrinia furnacalis*, widespread in Taiwan, was confirmed as (*Z*)-12-tetradecenyl acetate and its geometric isomer (*E*)-12-tetradecenyl acetate in a ratio of ca. 3:1 by gas chromatography and gas chromatography-mass spectrometry in selected ion monitoring mode. Males were attracted by the mixture of these two synthetic components in the field, but the attractiveness was less than by virgin females. The presence of minor components in the sex pheromone was therefore suggested.

Key Words—Lepidoptera, Pyralidae, *Ostrinia furnacalis*, sex pheromone, Asian corn borer, (*E*)- and (*Z*)-12-tetradecenyl acetate.

INTRODUCTION

The corn borer is an important pest of corn, peanut, and sorghum fields in Taiwan. From the external morphology, the Asian corn borer, *Ostrinia furnacalis*, is almost indistinguishable from the European corn borer, *Ostrinia nubilalis*. However, Mutuura and Munroe identified the species *Ostrinia furnacalis* as the Asian corn borer (Mutuura and Munroe, 1970; Cardé et al., 1978). The sex pheromone components from the Asian corn borer, *O. furnacalis* have been identified as (*E*)- and (*Z*)-12-tetradecenyl acetate. The ratio of these geometric

isomers *E* to *Z* was 53:47 (Cheng et al., 1981; Jiang and Klun, 1981) or 2:3 (Ando et al., 1980) in China and Japan, respectively. Those ratios are different from those of the sex pheromone components of the European corn borer, *O. nubilalis*, which is distributed widely in Europe and the United States (Kennedy and Anderson, 1980; Klun and Junk, 1977; Klun, 1975; Kochansky et al., 1975) and which has the same pheromone components.

Therefore, we attempted to determine the species and sex pheromone components of corn borer in Taiwan. We are specially interested in monitoring the population and/or development of mass trapping systems for this pest in the field. Female sex pheromones of nocturnal moths have been proved mostly to be unsaturated, straight-chain aliphatic acetates, alcohols, or the corresponding aldehydes. A classical approach to identify the insect sex pheromone involves the isolation of each component in pure form, and oxidative cleavage at the unsaturated center with ozone, followed by identification of the fragments by mass spectrometry. Those methods are tedious and difficult when the amount of material available is very small. Here we show that the corn borer in Taiwan is the Asian corn borer of which the pheromone is (*Z*)- and (*E*)-12-tetradecenyl acetate at a ratio of ca. 3:1 by gas chromatography and gas chromatography-mass spectrometry in the selected ion monitoring mode.

METHODS AND MATERIALS

Insect Materials. Larvae of the corn borer were reared successively on corn artificial diet at 25°C and relative humidity 70% under a 12-hr photoperiod. Female and male pupae were separated, and adult female moths were used for extraction of pheromone. For isolation of the pheromone, abdominal tips of 2-day-old virgin females were extracted with *n*-hexane. The crude preparations were stored at 0°C in a refrigerator until use.

Laboratory Bioassay for Monitoring Active Fractions. Male moths were kept under the same conditions as above until use. Two days after emergence, 10 males were placed in a 300 ml Erlenmeyer flask, and their behavior toward a piece of filter paper loaded with a sample was observed. The bioassay was carried out 9 hr after the light was turned off. When an active sample was introduced, the male moths showed hairpencil extension or wing vibration and quick flying followed by resting quietly at the filter paper.

Chemicals. The synthetic pheromones and standards were obtained from Dr. Tay-Yuan Chau, Department of Chemistry, National Chung-Hsing University, Tai Chung, Taiwan; and Shin-Etsu Chemical Co., Tokyo, Japan. All other chemicals were obtained from E. Merck Darmstadt, F.R. Germany.

Column Chromatography. Column chromatography with Florisil (60–100 mesh) was carried out according to a class-separating method for lipids (Carroll,

1961). Hexane, 5, 15, 25, and 50% ether in hexane, 2% methanol in ether, and 4% acetic acid in ether were successively used as eluting solvents.

High-Performance Liquid Chromatography. High-performance liquid chromatography was performed with a ERC-8710 high-pressure pump equipped with a Rheodyne model 7125 injector and ERC-8710 variable wavelength UV detector. The separation was carried out on a 30 cm \times 3.9 mm μ Porasil column eluted with 1% EtOAc in *n*-hexane at 0.8 ml/min flow rate and monitored at 235 nm.

Gas Chromatography. Gas chromatography was performed with a Hewlett-Packard model 5840A gas chromatograph equipped with a flame ionization detector, on-line to a Hewlett-Packard 5840 GC terminal. The packed column separation was carried out on a 3.05-m \times 2-mm ID, 3% OV-17 column (80°C for 2 min, then 10°C/min to 180°C then 5°C/min to 240°C, injection temperature = 200°C and detector temperature = 250°C). The separation was also carried out on a 50-m \times 0.32-mm fused silica capillary Carbowax 20 M column (60°C for 6 min then 15°C/min to 180°C then 4°C/min to 250°C, injection temperature 265°C and detector temperature 250°C); 55-m \times 0.32-mm fused silica capillary SP-2340 column (60°C for 6 min, 12°C/min to 180°C then 4°C/min to 240°C, injection temperature 245°C and detector temperature 250°C); 50-m \times 0.22-mm fused silica capillary CP Wax51 column (60°C for 6 min, 15°C/min to 180°C then 4°C/min to 240°C, injection temperature 265°C and detector temperature 250°C); and 25-m \times 0.32-mm fused silica capillary OV-1 column (60°C for 3 min, 10°C/min to 140°C then 4°C/min to 220°C, injection temperature 180°C and detector temperature 250°C).

Gas Chromatography-Mass Spectrometry. A Hewlett-Packard model 5985 mass spectrometer coupled with a gas chromatograph (model 5840A) and a mass data system (7900A) was used. The same GC capillary columns as listed under gas chromatography were used for separation. An electron impact ion source was used and the energy was set at 70 eV with the emission current at 300 μ A. The temperatures of column, injector, and detector were the same as under gas chromatography. The carrier gas was adjusted to a column head pressure of 10 psi.

Field Trapping. Field trapping of *O. furnacalis* by using synthetic materials was conducted in the field of Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI), Taichung, and Tainan District Agricultural Improvement Station (TDAIS), Tainan, during the flight season. A wing trap was used for this purpose. Synthetic materials were dissolved in *n*-hexane and loaded on an Aldrich rubber septum as bait. Two-day-old virgin females and unbaited traps were also used as references. The height of the trap above the ground usually was about 0.7–1 m. The lures in the traps were changed in a different direction every night over the experimental period; the recording was done every three or six nights.

RESULTS AND DISCUSSION

Florisil column chromatography of the crude extract from 2000 virgin females revealed that the active substances were eluted from the column by 5% ether in *n*-hexane (Table 1). The active substances were chromatographed on high-performance liquid chromatography, then fractionated by gas chromatography.

The sex pheromone of the corn borer was suggested to be either (*Z*)- and (*E*)-11-tetradecenyl acetate or (*Z*)- and (*E*)-12-tetradecenyl acetate for the European corn borer and the Asian corn borer, respectively (Cheng et al., 1981; Jiang and Klun, 1981; Kennedy and Anderson, 1980; Klun et al., 1980; Klun and Brindley, 1970; Nagai et al., 1977; Ren et al., 1983; Webster and Cardé, 1984). Separation of (*Z*)-7-, (*Z*)-9-, (*E*)-11-, (*Z*)-11-, (*E*)-12-, and (*Z*)-12-tetradecenyl acetate was successful on Carbowax 20 M, SP-2340, and CP Wax51 (Table 2).

Comparison of the chromatographic retention times of authentic tetradecenyl acetates on different capillary columns with the natural pheromone (Table 2) suggests that the corn borer in Taiwan is the Asian corn borer, *Ostrinia furnacalis*, where the sex pheromone is a mixture of (*E*)- and (*Z*)-12-tetradecenyl acetate.

For confirmation, analyses of the pheromone extract by gas chromatography-mass spectrometry (GC-MS) selected ion monitoring (SIM) were conducted with the fused silica CP Wax51 capillary column. The four fragment ions at $m/e = 254$ (M^+), 194 ($M^+ - \text{HOAc}$), 96, and 82 were selected for the

TABLE 1. BIOASSAY RESULTS OF THE FLORISIL FRACTIONS

Sample ^a	No. of males excited ^b
<i>n</i> -Hexane	0
5% Ether/ <i>n</i> -hexane	5
5% Ether/ <i>n</i> -hexane + <i>n</i> -hexane	4
5% Ether/ <i>n</i> -hexane + 15% ether/hexane	6
<i>n</i> -Hexane + 15% ether/ <i>n</i> -hexane	1
25% Ether/ <i>n</i> -hexane	0
50% Ether/ <i>n</i> -hexane	0
Crude extract	3

^a2 female equivalent.^b10 males in each flask as tested.

TABLE 2. RETENTION TIMES OF SEX PHEROMONE ANALOGS AND SEX PHEROMONE OF *O. furnacalis* ON VARIOUS GAS-LIQUID CHROMATOGRAPHIC COLUMNS

Column type ^a	Retention time (min)								Natural pheromone
	Z7-14:OAc	Z9-14:OAc	E11-14:OAc	Z11-14:OAc	E12-14:OAc	Z12-14:OAc			
Carbowax 20 M			21.30	21.50	21.60	21.90			21.60 21.90
SP-2340	18.72	18.74	18.69	18.93	18.88	19.21			18.86 19.19
CP Wax51	22.98	23.32	23.19	23.36	23.50	23.80			23.55 23.81
OV-1			14.70	14.75	15.08	15.09			15.14

^aSee Methods and Materials for GC conditions.

examination of the monoenyl acetates (Figure 1a). The ratio of *Z* and *E* isomers of the pheromone of our corn borer is about 3 by comparison with the area of each chromatograph of *Z* to *E* at each mass fragment of Δ -12-tetradecenyl acetate (i.e., 3370/1105, 2762/830, and 469/149 in Figure 1b). In Figure 1b, the

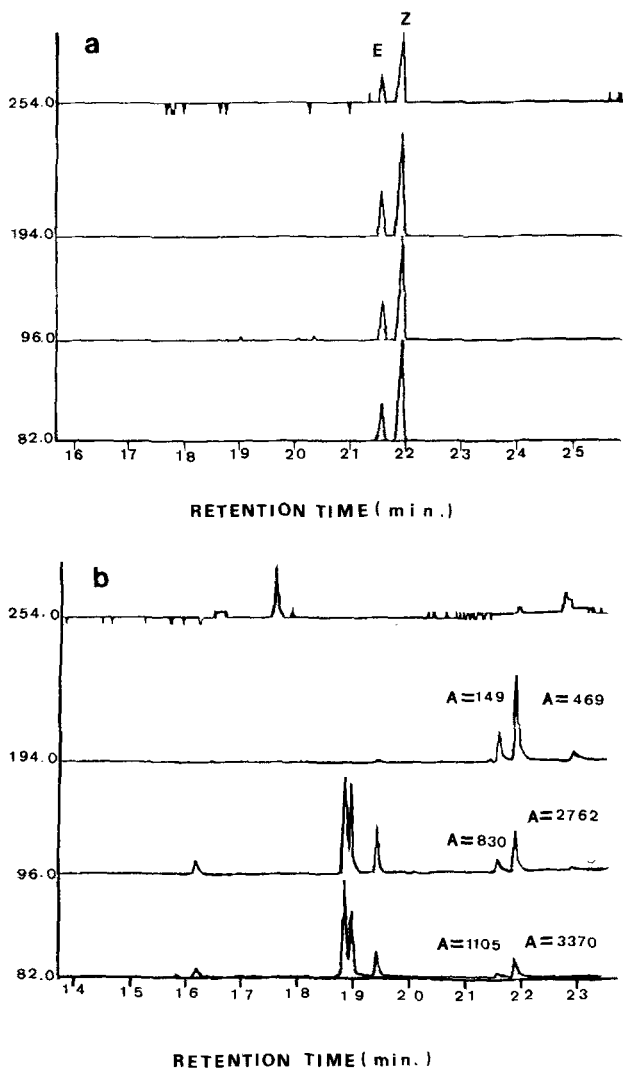


FIG. 1. Gas chromatogram (CP Wax 51 column) monitored by *m/e* = 254, 194, 96, 82, of (a) authentic (*E*)- and (*Z*)-12-tetradecenyl acetate and (b) extract from corn borer *O. furnacalis*.

GC-MS profile at 19–20 min showed some impurities, probably fatty acids. In search of lepidopteran sex pheromones, it has been shown that most pheromones identified from female moths are unsaturated straight-chain alcohols and/or their derivatives. By using a series of synthetic compounds, we may use the crude extract from the insect directly for GC-MS/SIM to identify the sex pheromone.

For further confirmation of the sex pheromone of *O. furnacalis*, field trials were carried out. The amount used for male attractiveness was in the 50- to 500- μ g range (Table 3). Four different compositions were tested, and results are summarized in Table 4. The best ratio of (Z)- to (E)-12-tetradecenyl acetate was 3 : 1, no male moths were caught with the combination of (Z)- and (E)-11-tetradecenyl acetate (unpublished result). These results coincide with the data obtained from GC-MS. The different ratios of (E)- and (Z)-12-tetradecenyl acetate of the sex pheromone of *O. furnacalis* at China, Japan, and Taiwan may be due to population differences. The numbers of males caught by the synthetic pheromone trap are far fewer than those caught by using two virgin females as

TABLE 3. NUMBERS OF MALE *O. furnacalis* IN STICKY TRAPS BAITED WITH VIRGIN FEMALES AND VARIOUS DOSAGES OF SYNTHETIC PHEROMONE, (Z)-12-TETRADECENYL ACETATE AND (E)-12-TETRADECENYL ACETATE (3 : 1) IN CORN FIELD AT TDAIS, TAINAN, TAIWAN

Bait used (μ g)	No. of males captured/trap ^a	
	12/1/85–1/31/86 ^b	5/1/86–6/1/86
2 virgin female moths ^c	254	322
1	0	
12.5	1	
25		
50	34	
100	65	
200		
400		
500	134	
1000		11
2000		19
4000		13
8000		16

^a Mean catches in four replicates over 60 or 30 successive nights of trapping and the duration of three nights for recording data. Data were transformed to $\sqrt{x + 1}$ for statistical analysis. The treatments were not significantly different from each other at 5% level.

^b Month/day/year.

^c Female moths (two days after emergence) without basic lure.

TABLE 4. CAPTURE OF MALE *O. furnacalis* IN WET STICKY TRAPS BAITED WITH SEX PHEROMONE OF DIFFERENT COMPOSITION

Sex pheromone component A:B ^a	No. male moths captured/trap ^b	
	12/11/85-2/13/86 ^c	11/1/85-1/31/86 ^b
2 virgin female	220	147
3:1	45	35
1:1	34	35
1:2	13	20
1:3	13	8
Unbaited	0	1

^aBasic lure: (Z)-12-tetradecenyl acetate (A) and (E)-12-tetradecenyl acetate (B), 50 µg total/trap.

^bMean catches in four replicates over the period of trapping and the duration of six nights for recording data. Data were transformed to $\sqrt{X + 1}$ before use and the treatments were not significantly different at 5% level.

^cTests were conducted in a corn field of TACTRI, Taichung, Taiwan.

^dTests were conducted in a corn field of TDAIS, Tainan, Taiwan.

a pheromone source. This fact clearly indicates that both (Z)- and (E)-12-tetradecenyl acetate are the major components of the pheromone, and the possibility of some minor components in the sex pheromone is also suggested. Research to identify the minor components and the broad-range ratios of (Z)- and (E)-12-tetradecenyl acetate in the field test is underway.

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OVIPOSITION STIMULANTS FOR THE BEETLE, *Monochamus alternatus* HOPE, IN INNER BARK OF PINE

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Abstract—Field and laboratory ovipositional responses of *Monochamus alternatus* Hope, respectively, to methanol and water extracts from pine inner bark were examined in comparison with those to pine inner bark, especially using a laboratory-built apparatus for the latter bioassay. Irrespective of the existence of volatiles from paraquat-induced lightwood, pine inner bark and its methanol and water extracts stimulated ovipositional response only in the presence of free moisture.

Key Words—*Monochamus alternatus* Hope, Coleoptera, Cerambycidae, pine inner bark, methanol extracts, water extracts, oviposition stimulants, lightwood.

INTRODUCTION

It has been well known that the cerambycid beetle, *Monochamus alternatus* Hope, is a vector of the pine wood nematode, *Bursaphelenchus xylophilus* Steiner et Buhrer, which causes severe mortality of pines (Morimoto and Iwasaki, 1972; Mamiya and Enda, 1972).

Recently, attractiveness of pines, treated with the herbicide paraquat, to *M. alternatus* has been confirmed (Yamasaki et al., 1980a). Paraquat induces an extensive area of oleoresin-soaked lightwood in the xylem of the pines (Roberts et al., 1973; Conley et al., 1977; Yamasaki et al., 1980b; Yamasaki and Sogo, 1982). The attraction of both sexes of *M. alternatus* arises from volatiles involved in the paraquat-induced lightwood (Yamasaki and Suzuki, 1982).

After landing on a dying (Katagiri et al., 1964) or paraquat-treated pine (Yamasaki and Sunago, 1983), a *M. alternatus* female moves to a site favorable

for oviposition, cuts a characteristic pit in the outer bark, inserts her ovipositor into the inner bark through the pit, and deposits eggs. In the case of the paraquat-treated tree, the site of oviposition is restricted within the area over the lightwood (Yamasaki et al., 1980a).

The role of chemical stimulants in the oviposition of *M. alternatus* is not known. This paper presents evidence that nonvolatile oviposition stimulants for *M. alternatus* occur in the pine inner bark.

METHODS AND MATERIALS

Field Test Materials. For lightwood, 17-year-old *Pinus densiflora* Sieb. et Zucc. trees were frill-treated with paraquat (1,1'-dimethyl-4,4'-dipyridinium dichloride) early in May 1986 (Yamasaki and Sogo, 1982). The paraquat-induced lightwood was harvested in mid-July 1986. Five lightwood samples (7 × 35 × 2.5 cm) were immediately used in the field, and the remainder was used for collection of volatiles.

For methanol extract, air-dried inner bark (0.9 kg) of *P. densiflora* trees of the same age, harvested late in April 1986, was milled and extracted with boiling methanol (2.8 liters) for 3 hr. Filtrate was concentrated under reduced pressure.

For impregnating filter paper, a sheet (21 × 35 cm) of 0.2-mm-thick filter paper (dry weight 6.459 g) was impregnated with 10 ml of the concentrate (19.38 w/v%) using a pipet, dried in air, in vacuo at 80°C for 12 hr in the presence of desiccating agents, and weighed. After standing in air for two days, the sheet containing 30% methanol extract was weighed again.

Field Bioassay. The bioassay was conducted from 8:00 PM on July 15 to 8:00 AM on July 16, 1986, in a stand where severe mortality of pines was caused by nematodes.

Four of the fresh lightwood samples were wrapped with a sheet of the test filter paper with or without methanol extract or a sheet (21 × 35 cm) of freshly harvested inner bark of *P. densiflora*. A tinfoil sheet (40 × 40 cm) to hold eggs was nailed to one of the two cross sections of the remaining lightwood sample without wrapper. After moistening the sheets of the test filter paper with a calculated volume of water, the baits with and without wrapper were hung on a wire 2 m above the ground at 5-m intervals along a ridge. The total number of *M. alternatus* attracted to each bait was counted at 8:30, 9:30, and 10:30 PM. The assayed wrappers were torn into pieces about 1 mm wide, and the number of eggs laid by *M. alternatus* was counted. To prevent hatching, eggs laid in parts awaiting examination were kept at 5°C.

Laboratory Test Materials. Lightwood volatiles (LW volatile) were collected by letting air from a cylinder pass through particles of the fresh lightwood and leading the gas into cooled traps (Yamasaki and Suzuki, 1982).

For test substrates, after being cut into 4×5 cm pieces in order to remove volatiles, bark, inner bark, and about 6-mm-thick outer bark of 17-year-old *P. densiflora* trees, harvested late in April 1986, were first dried in air for two months, then in an air-circulating oven at 80°C for seven days, in vacuo at 80°C for seven days, and weighed. Pieces of 4×5 -cm \times 1.5-mm-thick veneer made of commercial lumber of *P. densiflora* also were dried and weighed. In addition to these substrates, 4×5 -cm pieces of 1-mm-thick stainless steel were prepared.

For moistening these substrates, except the stainless steel, prior to laboratory bioassay, moisture content was achieved variously: (1) One series of 12 pieces was stood on an electronic balance, until the content reached 1.3%. (2) Another series to 5–6% by exposure to a cloud of steam. (3) A third to 18–56% by spraying with water, and, after 85–97% of a calculated water volume was adsorbed, the remainder was added using a syringe.

For fractions of water extract, the remainder (2 kg) of the fresh inner bark was reduced to particles and extracted with 3.9 liters of boiling water for 1.5 hr. The extract was fractionated as shown in Figure 1. Pieces of filter paper, 4×5 cm \times 0.7 mm thick, containing the 30% fraction I were prepared in the same way. Pieces of laminated filter paper, 4×5 cm and 1.1 to 1.3 mm thick, containing 30% fractions II, III, IV, or V were prepared as shown in Figure 2.

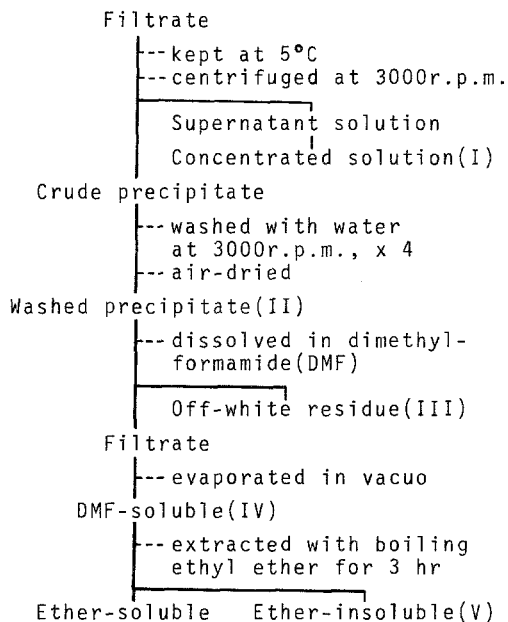


FIG. 1. Fractionation of water extract from fresh inner bark of *P. densiflora*.

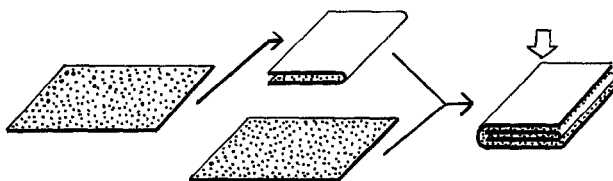


FIG. 2. Makeup of laminated filter paper containing powdery fraction II, III, IV, or V. Two 8.3×5 -cm pieces (dry weight 0.708 g for two) of 0.2-mm-thick filter paper were moistened with water (0.9 ml), and dusted with the sample (0.212 g). One piece was folded in two, and placed on the other. The other was folded back. This laminated filter paper with the sample was pressed under a pressure of 50 kg/cm^2 for 1 min.

The pieces were dried in vacuo at 80°C for 12 hr, weighed, and moistened with a calculated volume of water immediately before the laboratory bioassay.

The Insect. *P. densiflora* trees surviving where many other trees were dying were frill-treated with paraquat early in July 1986. About 200 *M. alternatus* females attracted to these trees were caught in mid-July and held in individual glass bottles (Yamasaki and Suzuki, 1982).

Laboratory Bioassay. The apparatus consisted of a hardware cloth chamber ($60 \times 7 \times 15$ cm) without one side (60×7 cm), a tin-plated chamber ($60 \times 7 \times 5$ cm) without one side (60×7 cm), and a hardware cloth screen (63×10 cm), as shown in Figure 3. The hardware cloth chamber was partitioned into eight compartments using tinplate sheets wrapped with hardware cloth.

A series of eight test pieces moistened with a calculated volume of water as described above was fastened on the hardware cloth screen with hairpins (Figure 3). LW volatile (0.5 ml) was placed into each of three Petri dishes (2.5

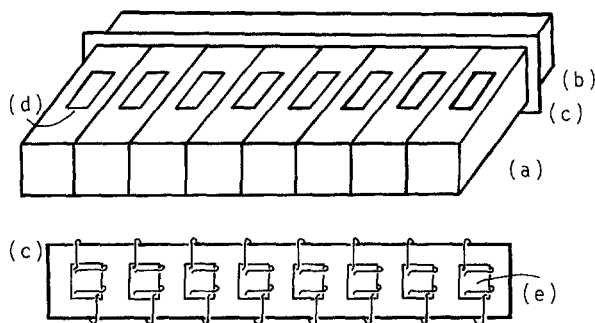


FIG. 3. Bioassay apparatus for *M. alternatus*: (a) compartment for each insect; (b) chamber in which LW volatile was placed; (c) hardware cloth screen; (d) insect entry hole (5×9 cm) with cover; (e) ovipositional substrates fastened on bed sheet (vener or stainless steel) with hairpins or wiring.

cm ID \times 1.8 cm) inside the tinplate chamber. The apparatus was assembled with wire. Individual *M. alternatus* females were placed into each of the eight compartments. The bioassay was conducted from 8:00 PM to 8:00 AM. As a control, bioassay in the absence of LW volatile was conducted in another room (about 100 m away). The surface of the assayed materials was observed, and the number of eggs laid was counted. The percentage of ovipositional response was calculated as follows: Ovipositional response (%) = $100 \times \text{No. which responded} / 8 \text{ individuals}$.

RESULTS

Field Attraction and Oviposition of M. alternatus. Both sexes of *M. alternatus* were attracted to every bait in the field (Table 1). This table shows that at least one or two females were attracted to each of the baits (e.g., two females to the bait without wrapper). Four and 25 eggs laid by *M. alternatus* were present in or under the moistened filter paper with methanol extract and the fresh inner bark, respectively. It is impossible to compare the numbers of eggs (4 vs. 25) because the real number of the females attracted to each of the baits is not shown in this table. No eggs, however, were observed at the other baits; with that the two former observations are comparable.

Oviposition of M. alternatus in Laboratory Affected by Free Moisture. Figure 4 shows the effect of moisture content of pine inner bark at the onset of

TABLE 1. FIELD ATTRACTION AND OVIPOSITION OF *M. alternatus*

Bait	Sex	Total no. of <i>M. alternatus</i> attracted to each bait			Eggs laid (N)
		8:30 PM	9:30 PM	10:30 PM	
Lightwood (a) ^a	Female	1	2	2	0
	Male	1	4	2	
Lightwood (a) + moistened filter paper (b)	Female	0	1	1	0
	Male	1	2	2	
Lightwood (a) + air-dried filter paper containing 30% MeOH extract (c)	Female	1	0	1	0
	Male	2	3	0	
Lightwood (a) + moistened filter paper containing 30% MeOH extract (b)	Female	1	1	0	4
	Male	1	0	2	
Lightwood (a) + fresh inner bark (d)	Female	0	1	2	25
	Male	1	2	1	

^aMoisture content: a, 20-35%; b, 50%; c, 9%; d, 67%.

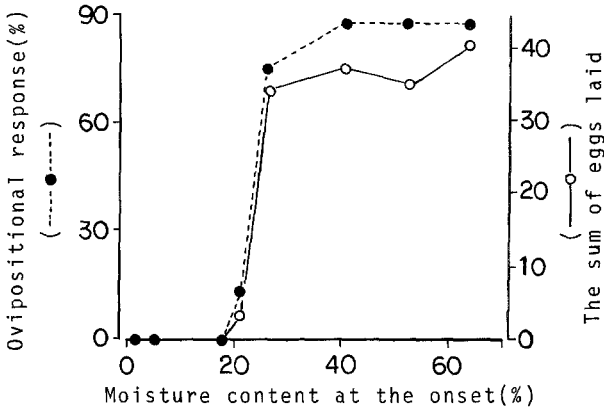


FIG. 4. Effect of moisture content of inner bark of *P. densiflora* on oviposition by *M. alternatus*. The laboratory test was conducted July 18–19, 1986, using LW volatile. Veneer with moisture content almost equivalent to that of the corresponding inner bark was used as a bed sheet. When fresh inner bark with 64% moisture was assayed, veneer with 56% moisture was used. Moisture contents of eight series of another four pieces which were placed outside their respective apparatus for 12 hr were substituted for those of inner bark after bioassay.

bioassay on oviposition by *M. alternatus*. Moisture contents of 1.3 and 5.0% at the onset increased to a level of 13.6–14.6% overnight. The contents of 18.0 and 21.0% decreased to levels of 16.4–17.0% and of 16.9–19.1%, respectively. The contents of 26.0% and more also decreased to a level of 21.7–26.4%. When inner bark with 18.0% or less moisture at the onset was assayed, neither egg laying nor the slight scoring of the inner bark prior to ovipositor insertion was observed, although a few scars produced by gnawing or feeding beetles were observed. Under inner bark with 21.0% moisture, three eggs were laid by one of eight beetles. Thirty-four eggs were laid in or under inner bark with 26.0% moisture by six of eight beetles, and in or under inner bark with 41.0% or more moisture 35–40 eggs were laid by seven of eight beetles. Besides the scars, openings resulting from ovipositor insertion, like pinholes of less than 1 mm ID in the respective scores also were observed in the assayed inner bark with 21.0% and more moisture.

Oviposition of M. alternatus in Various Substrates. Materials tested in the laboratory are shown in Table 2. Bed sheet alone and filter paper with or without the bed sheet did not induce oviposition by *M. alternatus*. Scars different from those on inner bark were left on the filter paper, but were not observed on the bed sheet alone. Outer bark containing 5–6% moisture elicited no oviposition.

TABLE 2. LABORATORY OVIPOSITION OF *M. alternatus* IN VARIOUS SUBSTRATES WITH AND WITHOUT VOLATILES (JULY 22-25, 1986)

Substrate	LW volatile present ^a		LW volatile absent ^a	
	Ovipositional response (%)	Eggs laid	Ovipositional response (%)	Eggs laid
Veneer	0	0	0	0
Stainless steel	0	0	0	0
Filter paper	0	0	0	0
Filter paper + veneer	0	0	0	0
Filter paper + stainless steel	0	0	0	0
Outer bark ^b + veneer ^b	0	0	0	0
Outer bark + veneer	0	0	12.5	2
Outer bark + stainless steel	12.5	2	0	0
Inner bark	87.5	42	75	42
Inner bark + veneer	87.5	49	87.5	39
Inner bark + stainless steel	87.5	38	87.5	40
Bark + veneer	87.5	53	100	38
Bark + stainless steel			75	42

^aVolatiles extracted from paraquat-induced lightwood.

^bMoisture content at the onset was 5-6%; all others were 49-56%, except stainless steel.

On the other hand, when outer bark with 49-56% moisture was assayed, one of eight beetles laid two eggs; otherwise, no eggs were observed. Pits, slots, or galleries of various sizes in every series of the outer bark were 9-13 in number. In contrast to these results, even in the absence of LW volatile, 42, 39, and 40 eggs were laid in or under moistened inner bark by six, seven, and seven of eight beetles, respectively. In or under moistened bark 53, 38, and 42 eggs were laid by seven, eight, and six of eight beetles, respectively. On average, 21 pits were left on a series of eight bark pieces.

Oviposition of M. alternatus in Laboratory Affected by Fractions of Water Extract. Five fractions of water extract were assayed (Table 3). Beside scars, small openings were observed in every series, except dried filter paper containing fraction IV, which elicited no oviposition. One of eight beetles laid two or three eggs under moistened filter paper with fraction I. One beetle laid four eggs under moistened filter paper with fraction III in the presence of LW volatile. On the other hand, moistened filter paper with fractions II, IV (Figure 5), and V elicited ovipositional responses of 75% of the 17 or 20 eggs laid, 87.5% of 21 or 18; 87.5% of 20; and 75% of 24, respectively.

TABLE 3. EFFECT OF FRACTIONS OF WATER EXTRACT ON OVIPOSITIONAL RESPONSE OF *M. alternatus* (JULY 26-28, 1986)

Material with veneer ^a	LW volatile present		LW volatile absent	
	Ovipositional response (%)	Eggs laid	Ovipositional response (%)	Eggs laid
Filter paper containing 30% fraction I	12.5	3	12.5	2
Laminated filter paper containing 30% fraction II	75	17	75	20
Laminated filter paper containing 30% fraction III	12.5	4	0	0
Laminated filter paper (a) containing 30% fraction IV	0	0	0	0
Laminated filter paper containing 30% fraction IV	87.5	21	87.5	18
Laminated filter paper containing 30% fraction V	87.5	20	75	24
Inner bark	87.5	42	87.5	38

^aMoisture contents of materials and veneer at the onset, respectively, were 55 and 50-56%, except the case of (a); in this case filter paper material and veneer, respectively, containing 6% and 5% moisture were used.

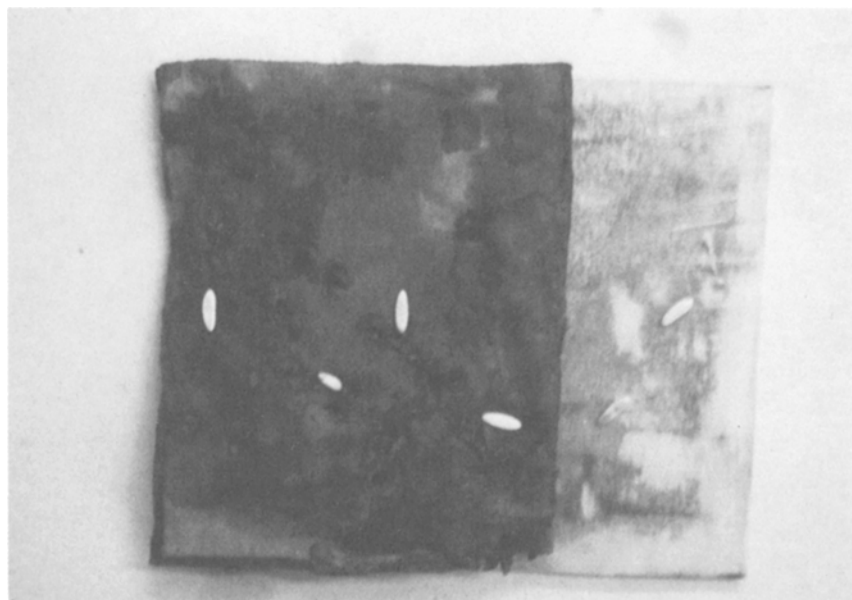


FIG. 5. Six eggs laid under moistened filter paper with fraction IV by a *M. alternatus* female. The assayed substrate is turned inside out and placed on veneer. This beetle laid two other eggs in the substrate. Openings resulting from ovipositor insertion also are shown.

DISCUSSION

It is evident from Tables 1–3 that oviposition of *M. alternatus* is not evoked by volatiles, but by nonvolatiles occurring in the inner bark of *P. densiflora*. The oviposition stimulants are extractable in methanol, dimethyl formamide, or water, but are insoluble in ethyl ether. The ovipositional response elicited by fraction II, IV, or V was equivalent to 86–100% of that elicited by inner bark as a control, whereas the number of eggs laid was only 40–60% of that laid in the inner bark (Table 3).

The bed sheet was found to be unnecessary for oviposition (Table 2), but it is convenient to prevent the loss of eggs laid. Oviposition of *M. alternatus* appears to be independent of cambium or xylem of host pine.

It has been known that in the sphingid *Manduca sexta* L. (Sparks, 1973) and noctuid *Anadevidia peponis* F. (Ichinose and Sasaki, 1975) moisture itself evokes oviposition. In *M. alternatus*, however, free moisture must be present in order for the chemical stimulants from inner bark to elicit oviposition (Figure 4, Tables 1 and 3). Critical moisture content necessary for oviposition appears to be between 18% and 21% under these experimental conditions. Contact chemoreception through intervention of free moisture has been confirmed in some butterflies such as *Byasa alcinous* K., *Leudhorfia japonica* L., *Papilio xuthus* L., *P. machaon hippocrates* C. et F., *P. macilentus* J. (Nishida, 1977), *P. protenor demetrius* C. (Ichinose and Honda, 1978), and *P. bianor dehaanii* C. et F. (Abe et al., 1981). In *M. alternatus*, however, chemoreceptors have not been identified.

The behavior of cutting a pit in the outer bark prior to ovipositor insertion was independent of moisture content. Moisture content of the outer bark of a living pine varies with atmospheric conditions, unlike that of inner bark. It was alluded that volatiles from bark of dying pines evoke this behavior (Yamane et al., 1975). In this study, however, the behavior of cutting a pit in outer bark was observed even in the absence of LW volatile. On inner bark with free moisture, the behavior of slightly scoring it prior to ovipositor insertion was observed. On filter paper with fractions I to V of water extract, no pit-making behavior was observed. Nonvolatiles in outer bark may induce this behavior. In the absence of LW volatile, fraction III elicited no oviposition (Table 3), but 10 openings were produced by four of eight beetles. Ovipositor insertion appears to be induced by a wide variety of factors, in addition to oviposition stimulants, involved in pine inner bark.

The present results suggest the feasibility of controlling *M. alternatus* by using oviposition stimulants in combination with attractants produced by paraquat-induced lightwood without insecticide application.

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RECEPTOR CHIRALITY AND BEHAVIORAL
SPECIFICITY OF THE BOLL WEEVIL, *Anthonomus
grandis* Boh. (COLEOPTERA: CURCULIONIDAE),
FOR ITS PHEROMONE, (+)-GRANDISOL

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Abstract—Electrophysiological recordings from antennal olfactory receptors and field behavioral experiments showed both male and female boll weevils, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), to respond specifically to (+)-grandisol, an enantiomer of compound I of the boll weevil aggregation pheromone. Single-cell recordings revealed antennal olfactory neurons in both male and female weevils keyed to (+)-grandisol. Electroantennograms in response to serial dilutions of the grandisol enantiomers showed a threshold 100 to 1000 times lower for (+)-grandisol relative to its antipode. In field behavioral experiments, both sexes were significantly more attracted to (+)-grandisol in combination with the three other pheromone components than the combination with (–)-grandisol. When (–)-grandisol was placed with the (+)-enantiomer at equal dosages, a slight although statistically insignificant inhibition occurred. Subsequent field tests showed that the low level of attraction exhibited by (–)-grandisol in combination with the other three pheromone components could be attributed to the other three components alone. These results are in contrast with an earlier study, which found (–)-grandisol to be as attractive as the (+)-enantiomer.

Key Words—Boll weevil, olfaction, receptor cell, *Anthonomus grandis*, Coleoptera, Curculionidae, enantiomer, grandisol, chirality, electroantennogram, aggregation pheromone, neurobiology, structure-activity.

INTRODUCTION

The importance of chirality in insect chemical communication is well known (Silverstein, 1979; Mori, 1984). Sensitivity and specificity in the detection and translation of pheromonal messengers by insects are especially crucial since chiral chemical cues may be important in their orientation to conspecifics for aggregation and/or mating. In general, insects that produce a chiral pheromone detect and respond behaviorally to the predominant enantiomer or blend of enantiomers released (Iwaki et al., 1974; Borden et al. 1976, 1980; Hedden et al., 1976; Klimetzek et al., 1976; Vité et al., 1976a,b; Wood et al., 1976; Yamada et al., 1976; Birch et al., 1977, 1980; Harring and Mori, 1977; Kra-wielitzki et al., 1977; Miller et al., 1977; Lanier et al., 1980; Levinson and Mori, 1980; Mustaparta et al., 1980; Payne et al., 1982; Dickens et al., 1985; Kodama et al., 1987). However, the antipode of the insect's pheromone may be released by a sympatric species or a different population of the same species and may inhibit response to the pheromone (Vité et al., 1976b; Light and Birch, 1979).

Male boll weevils, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), release in their frass an aggregation pheromone that consists of four components: I, (+)-*cis*-2-isopropenyl-1-methylcyclobutane ethanol; II, *cis*-3,3-dimethyl- $\Delta^{1,\beta}$ -cyclohexane ethanol; III, *cis*-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde; and IV, *trans*-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde (Tumlinson et al., 1969). Although one of these pheromone components, I (grandisol), is produced as the (+)-enantiomer by the insect, its antipode, (-)-grandisol, was shown to have biological activity equal to the isolated pheromone component (Mori et al., 1978).

The biological activity of (-)-grandisol (Mori et al., 1978) has been an anomaly in insect pheromone biology. This remains as one of only two reports in which an insect produced one enantiomer of a compound as its pheromone, and its antipode, once synthesized, had equal biological activity (Mori et al., 1978, 1981). Although Silverstein (1979) reported that no instances had been documented in which an insect produced only a single enantiomer and could not distinguish between it and its antipode, he did cite earlier work in which humans were sometimes unable to distinguish between enantiomers (Lensky and Blum, 1974).

The purpose of the experiments reported here was to investigate detection of the enantiomers of grandisol by the boll weevil through electrophysiological recordings from the antennal receptors. In addition, due to results of the electrophysiological experiments, which indicated a lack of detection of (-)-grandisol, quantitative behavioral experiments were done in the field to reinvestigate previously reported biological activity of the antipode of the pheromone component (Mori et al., 1978).

METHODS AND MATERIALS

Insects. Adult *A. grandis* used in these experiments were obtained from a small laboratory colony annually infused with feral insects maintained at the USDA-ARS Boll Weevil Research Unit. Upon emergence, insects were sexed and fed cotton squares. For electrophysiological studies, groups of five insects of the same sex were maintained on moist filter paper in Petri dishes until use following receptor maturation at four days postemergence (Dickens and Moorman, 1987). Insects used in these studies were 4–7 days of age. For field experiments, groups of 50 insects of the same sex were held in paper cartons (ca. 0.5 liter) with screen tops. Insects used in the field releases were 6–13 days postemergence. All insects were held in incubators at 26°C under a photoregime of 16 hr of light (ca. 700 lux) and 8 hr of darkness.

Chemicals. Optical isomers of grandisol were 100% optically and chemically pure as prepared by Mori and Miyake (1987). Racemic grandisol, II, and III + IV (a 50:50 mixture), each >95% chemical purity, were obtained from Albany International, Controlled Release Division, Buckeye, Arizona. All compounds were prepared as 10 $\mu\text{g}/\mu\text{l}$ dilutions in hexane. Serial dilutions of (+)- and (-)-grandisol were prepared in hexane from 0.01 to 10 $\mu\text{g}/\mu\text{l}$ for electrophysiological studies. From these dilutions, a 50:50 mixture of the enantiomers was also prepared. 1-Hexanol, used as a standard in electroantennogram (EAG) experiments, was 98% chemically pure as obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.

Electrophysiology. Single-cell recording techniques are described in detail elsewhere (Dickens, 1979; Dickens et al., 1984) and were essentially a modification of an earlier technique (Boeckh, 1962). In brief, microelectrodes used for recordings were constructed from 50.8- μm -diameter tungsten wire electrolytically sharpened to a tip of 1–2 μm . The recording electrode was positioned under optical control (150–200 \times) with a Leitz high-power micromanipulator near either the base of one of the three sensory bands encircling the club or the base of an individual sensillum just distal to each sensory band region. The ground electrode was inserted in either the rostrum or distal end of the pedicel. Action potentials were amplified and conditioned by a Grass P-15 AC preamplifier and Tektronix 5A22N amplifier prior to visualization on a Tektronix 5223 digitizing oscilloscope. The signal then passed through a Teac R51-D data recorder for recording on cassette tapes prior to visualization on a Tektronix 5111 analog storage oscilloscope. Data were photographed from the analog oscilloscope with a Tektronix C-5C oscilloscope camera and Polaroid film. Trains of action potentials were counted either visually or by a Frederick Haer slope height window discriminator and timed counter. Single-cell responses were recorded from 12 males and 7 females. One or two spike heights were observed for each preparation.

EAGs were recorded as previously described (Dickens, 1984). Ag-AgCl capillary electrodes filled with physiological saline (Oakley and Schafer, 1978) were used. Following prepuncture with a sharpened tungsten needle, the recording electrode was inserted in the distal end of the club; the ground electrode was inserted into the distal end of the pedicel. The signal was then amplified by a Grass P-16 DC preamplifier prior to visualization on a Tektronix 5111 analog oscilloscope and recording for storage with a strip-chart recorder.

Serial dilutions of grandisol were delivered as 1- μ l aliquots placed on filter paper (8 \times 18 mm) inserted into glass cartridges (80 mm \times 5 mm ID) oriented toward the preparation from a distance of 1 cm. Molecules evaporating from the filter paper were carried over the preparation by hydrocarbon-free air, which had been filtered and dried. Serial dilutions were delivered from the lowest to the highest concentration. Stimulus duration was 0.5 sec and 1 sec for single-cell and EAG preparations, respectively. Air flow was 1 liter/min. More than 3 min were allowed between EAG stimulations, while 2-3 min elapsed between presentations of stimuli to single cell preparations. These time intervals were adequate for recovery of both EAG and single cell activity.

In EAG studies, three replicates were recorded for serial dilutions of each grandisol enantiomer and mixture of enantiomers for both male and female *A. grandis*. 1-Hexanol at the 100- μ g dosage was used: (1) to normalize all responses; (2) to ensure viability and constancy of the preparation; and (3) to allow for relative comparisons with previously recorded species (Dickens, 1984; Dickens and Boldt, 1985; Dickens et al., 1986; Glancey and Dickens, 1988; Light et al., 1988). Stimulation with the standard both preceded and followed each serial dilution level. Responses to the enantiomers at a given dosage were expressed as a percent of the mean of the two nearest responses to the standard (Dickens, 1981, 1984). The threshold of response in the EAG studies was considered to be the dosage at which the standard error of the mean of the response was not equal to or less than zero (Dickens, 1984).

Field Experiments. Compounds were placed in 0.5-dram screw-cap vials as 10- μ l aliquots of 10 μ g/ μ l dilutions of the pheromone components using glass capillaries. These vials were then sealed with Teflon-lined screw caps and placed in a cooler until use in the field. Grandisol was always kept in a separate vial. Upon reaching the field, screw caps were removed from the vials and both caps and vials were placed in the top of an aluminum film container (3 cm diam. \times 4.3 cm height) that had five holes (0.23 cm diam.) drilled near its outer edge. The bottom of the can was then screwed onto the top, and the inverted container was then placed in the trap beneath the screen funnel.

The experimental design consisted of placing four traps (Mitchell et al., 1976), one at each corner of a 20-m square, in an open, isolated area. The two treatments being tested were placed alternately and thus were located adjacent each other at the corners of the square. One hundred insects (50 males and 50

females) were then released 20 m from each side of the square at ca. 1400 hr (400 insects total for each replicate of each experiment). Traps were then immediately baited. Each trap was checked and the weevils removed on the day of release at 1500 hr, 1600 hr, and 1800 hr, and the following day at 1500 hr and 1600 hr prior to removal of the baits at 0830 hr the next day.

Three replicates of each of the following three treatment combinations were tested: (1) (+)-grandisol (400 μg), II (300 μg), III + IV (100 μg) vs (-)-grandisol (400 μg), II (300 μg), III + IV (100 μg); (2) (+)-grandisol (400 μg), II (300 μg), III + IV (100 μg) vs (+)-grandisol (400 μg), and (-)-grandisol (400 μg), II (300 μg), III + IV (100 μg); and (3) (\pm)-grandisol (400 μg), II (300 μg), III + IV (100 μg) vs II (300 μg), III + IV (100 μg).

Statistical Analyses. Trap catches for simultaneous paired treatments in the field experiments were compared using a paired *t* test (Ostle, 1963).

RESULTS

Electrophysiology. Of 43 neurons recorded from male and female *A. grandis*, three were reliably activated by racemic grandisol. Optical isomers of grandisol were available for stimulation of two of these neurons (one male and one female), which were both activated by (+)-grandisol (Figure 1). Responses increased with increasing dosages (Figure 2). While an intermediate response was given to the mixture of enantiomers, significant responses were elicited by (-)-grandisol only at very high dosages.

Results from the EAG studies were similar to those obtained in the single-cell recordings. Both male and female weevils had a threshold between 0.1 and

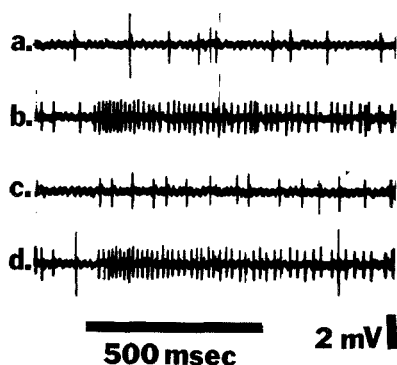


FIG. 1. Activity of a single cell in the medial sensory band of an *A. grandis* female. Spontaneous activity (a). Response to volatiles emanating from: (b) 1 μg of (+)-grandisol; (c) 1 μg of (-)-grandisol; (d) 1 μg of a 50:50 mixture of (+)- and (-)-grandisol. Horizontal line represents duration of stimulus (500 msec). Vertical bar is 2 mV.

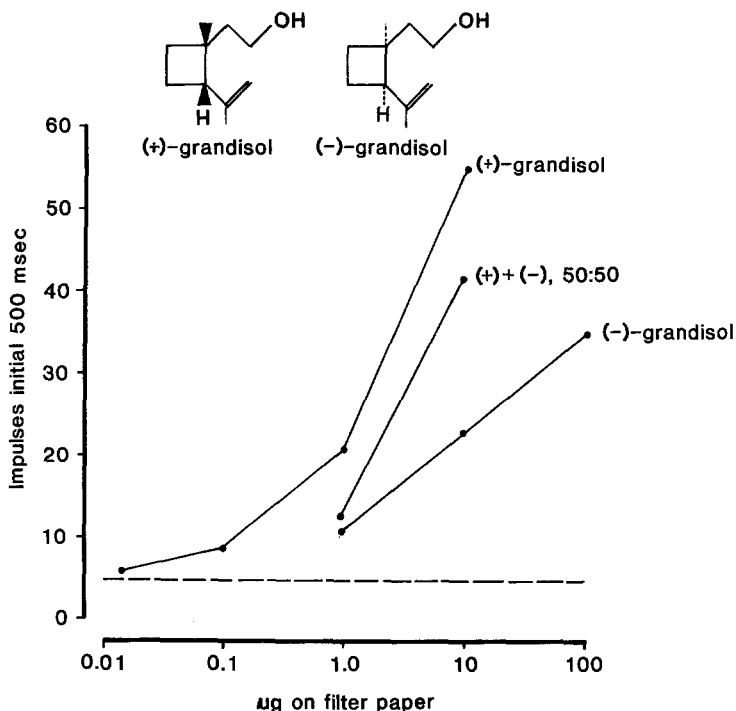


FIG. 2. Dosage-response data obtained from a cell in the medial sensory band of an *A. grandis* male in response to the enantiomers of grandisol. Broken horizontal line represents spontaneous activity level.

1.0 μg on filter paper for (+)-grandisol (Figure 3a and b). An intermediate response was elicited by the mixture of enantiomers, while significant EAGs were elicited by the (-)-enantiomer only at 100 μg .

Field Experiments. Competitive field experiments in which the grandisol enantiomers were each paired with the three other pheromone components clearly demonstrated the combination with the (+)-enantiomer to be the most attractive ($P < 0.01$) (Table 1).

To further demonstrate the inactivity of the (-)-enantiomer, it was paired with the (+)-enantiomer and placed in competition with the (+)-enantiomer alone, each treatment in combination with the other three pheromone components. This experiment showed no significant difference between the combination of enantiomers and the (+)-enantiomer alone. If the (-)-enantiomer were active, one might expect a greater response to the combination of enantiomers since a larger quantity was released. This was not the case. Indeed, a smaller,

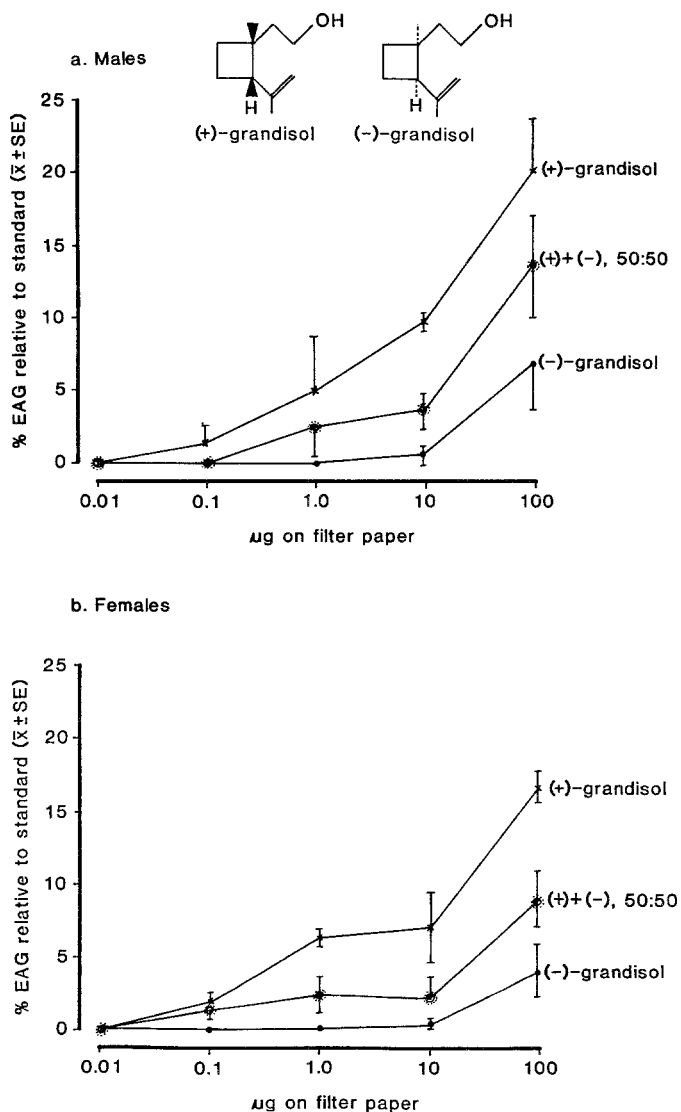


FIG. 3. Dosage-response curves constructed from EAGs of male (a) and female (b) *A. grandis* to serial dilutions of (+)-grandisol, (-)-grandisol, and a 50:50 mixture of (+)- and (-)-grandisol. Each point represents a mean from three replicates. Vertical bars represent standard errors.

Table 1. Mean (\pm SE; $N = 3$) Response of *Anthonomus grandis* in Competitive Field Tests with Enantiomers of Grandisol

Treatment pair	\bar{X}^a (\pm SE) capture/replicate	Sex ratio M:F
(+)-I, II, III + IV	35** (\pm 8.02)	0.85:1
vs.		
(-)-I, II, III + IV	2.67 (\pm 2.67)	1.25:1
(+)-I, II, III + IV	27 (\pm 7.00)	1.16:1
vs.		
(+)-I, (-)-I, II, III + IV	21.67 (\pm 4.33)	1.60:1
(\pm)I, II, III + IV	22.67* (\pm 6.96)	1.38:1
vs.		
II, III + IV	2 (\pm 1.15)	5.00:1

^a * $P < 0.05$; paired t test; ** $P < 0.01$; paired t test.

although not significantly smaller, number of weevils were captured by the combination of enantiomers.

A third field experiment was done to determine if the few weevils captured by the combination of (-)-grandisol and II, III + IV in the first experiment might be attributable to the attractiveness of II and III + IV alone. In this experiment, the number of weevils captured by II and III + IV alone was significantly less than the combination with racemic grandisol and was nearly identical to the number captured by II and III + IV with (-)-grandisol in the first experiment.

DISCUSSION

The greater activity of (+)-grandisol relative to its antipode is clearly demonstrated by our results. In fact, competitive field experiments involving (+)-grandisol vs (-)-grandisol or (+)- and (-)-grandisol in combination with the other three pheromone components indicate very little attraction by (-)-grandisol and slight, although insignificant, inhibition when placed in combination with the (+)-enantiomer (Table 1). Results of a third field experiment indicated that the attractiveness of (-)-grandisol, II, and III + IV could be attributable to II and III + IV alone. These results are in contrast with results obtained in an earlier study, which indicated (-)-grandisol to be as attractive as the naturally occurring pheromone component, (+)-grandisol (Mori et al., 1978). Results of this earlier study might be explained by the fact that: (1) the enan-

tiomers used were not 100% optically pure, and (2) conclusions concerning the activity of the enantiomers were based only on a few "no choice" laboratory behavioral bioassays. Mori et al. (1978) mention that the enantiomers were not 100% optically pure, and results of the current field tests and earlier laboratory bioassays (Tumlinson et al., 1969) indicate the combination of II, III, and IV to be somewhat attractive alone.

Our electrophysiological data correlate well with the field behavioral data. Both male and female *A. grandis* had a 100- to 1000-fold lower threshold for (+)-grandisol relative to its antipode (Figure 3). EAGs elicited by the mixture of enantiomers were intermediate to the two individual enantiomers at each dosage. Dosage-response curves for each enantiomer and the mixture of enantiomers were parallel. These results indicate that (-)-grandisol at a very high dosage might stimulate either receptors for the (+)-enantiomer or other less specific receptors. Our current single-cell data support the former notion that (-)-grandisol at a very high dosage can stimulate cells primarily driven by the (+)-enantiomer (Figure 2).

Although our data support the conclusion that (-)-grandisol is inactive for the population of boll weevils occurring in our region, variation in both the production of and response to enantiomers of aggregation pheromone components occurs in different populations of other Coleoptera, e.g., *Ips pini* (Birch et al., 1980; Lanier et al., 1980). Eastern *I. pini* produce and respond to a 65:35 blend of (+)- and (-)-ipsdienol, while the western population produces and responds to only (-)-ipsdienol and is interrupted by the (+)-enantiomer. Receptor cells keyed to each enantiomer occurred in individuals from each population, although they occurred in different proportions (Mustaparta et al., 1980). Since only three grandisol cells have been recorded to date, and the enantiomers of grandisol were available for stimulation of only two of them, our results are too preliminary to conclude the nonexistence of (-)-grandisol cells. Furthermore, we have not yet investigated other populations of boll weevils. However, additional electrophysiological and behavioral studies are already in progress on boll weevil populations in Arizona, Mexico, and South Carolina, and investigations are planned on the receptor system of another curculinoid genus, *Pissodes*, which also uses grandisol as an aggregation pheromone component (Booth et al., 1983). Also, in light of the current study, the other reported example, in which the antipode of the German cockroach pheromone was as active as the naturally occurring compound (Mori et al., 1981), deserves reinvestigation. Synthetic attempts to prepare 100% optically pure German cockroach pheromone will commence immediately.

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DIFFERENTIAL RECOGNITION OF GEOMETRIC ISOMERS BY THE BOLL WEEVIL, *Anthonomus grandis* BOH. (Coleoptera: Curculionidae): EVIDENCE FOR ONLY THREE ESSENTIAL COMPONENTS IN AGGREGATION PHEROMONE

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Abstract—For two decades, the aggregation pheromone of the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), was thought to consist of four compounds: I [(+)-(Z)-2-isopropenyl-1-methylcyclobutane ethanol]; II [(Z)-3,3-dimethyl- $\Delta^{1,\beta}$ -cyclohexane ethanol]; III [(Z)-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde]; and IV [(E)-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde]. Evidence is presented from behavioral and electrophysiological studies to show that only three of these components, I, II, and IV, are essential for attraction. Competitive field tests, in which each possible three-component blend was tested against the four-component mixture, demonstrated that omission of I, II, or IV resulted in decreased trap captures ($P < 0.01$). Trap captures by these blends lacking I, II, or IV resembled those by the hexane solvent alone in a similar experiment. However, omission of III did not significantly alter field attractiveness of the blend. Dosage-response curves constructed from electroantennogram responses of both males and females to serial dilutions of III, IV, and a 50:50 mixture of the geometric isomers III and IV showed both sexes to be 10- to 100-fold more sensitive to IV than III. Data from the electrophysiological studies were consistent with a single acceptor type for the (E)-cyclohexylidene aldehyde, IV, for males, and possibly one or two acceptor types for III and IV for females. Possible roles for the (Z)-cyclohexylidene aldehyde, III, and implications for the pheromonal attractant currently used in boll weevil eradication/suppression programs are discussed.

Key Words—Boll weevil, *Anthonomus grandis*, aggregation pheromone, multicomponent, behavior, electroantennogram, neurobiology, structure-activity, geometric isomers, cotton, Coleoptera, Curculionidae.

INTRODUCTION

The boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), is a pest of cotton, *Gossypium hirsutum* L. in the United States and Central and South America (Cross, 1983). It is narrowly oligophagous, feeding primarily only on cotton and its close relatives in the family Malvaceae (Cross et al., 1975). Once male boll weevils locate their host plant, feeding ensues, and they release in their frass an attractant pheromone (Cross and Mitchell, 1966; Keller et al., 1964). This pheromone aggregates both sexes at various times of year (Hardee et al., 1969, 1972) and consists of four components: I [(+)-(Z)-2-isopropenyl-1-methylcyclobutane ethanol]; II [(Z)-3,3-dimethyl- $\Delta^{1,\beta}$ -cyclohexaneethanol]; III [(Z)-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde]; and IV [(E)-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde) (Tumlinson et al., 1969) (Figure 1). The identification of this multicomponent pheromone was based on laboratory behavioral bioassays (Tumlinson et al., 1969) with its activity in nature verified by subsequent field experiments (Hardee et al., 1972).

Since the identification of the boll weevil aggregation pheromone nearly two decades ago (Tumlinson et al., 1969), and the subsequent report of lack of chiral specificity for I (grandisol) (Mori et al., 1978), research on the pheromone has focused on the determination of attractive ratios of its four components (Hardee et al., 1974), and the development of formulations for use in the field (McKibben et al., 1971, 1980). In contrast to earlier work (Mori et al., 1978), we recently showed that boll weevils respond behaviorally only to (+)-grandisol and possess olfactory cells specific for this enantiomer, while (-)-grandisol is inactive or slightly inhibitory (Dickens and Mori, 1989). This discovery prompted us to reinvestigate the activity of each of the boll weevil pheromone components (Tumlinson et al., 1969). We report here the results of our electrophysiological and behavioral experiments.

METHODS AND MATERIALS

Insects. Adult *A. grandis* used in these experiments were obtained from a small laboratory colony annually infused with feral insects maintained at the USDA-ARS Boll Weevil Research Unit. Upon emergence, insects were sexed and fed cotton squares. For field experiments, groups of 50 insects of the same sex were held in paper cartons (ca. 0.5 liter) with screen tops. Insects used in the field releases were 5–14 days postemergence. For electrophysiological stud-

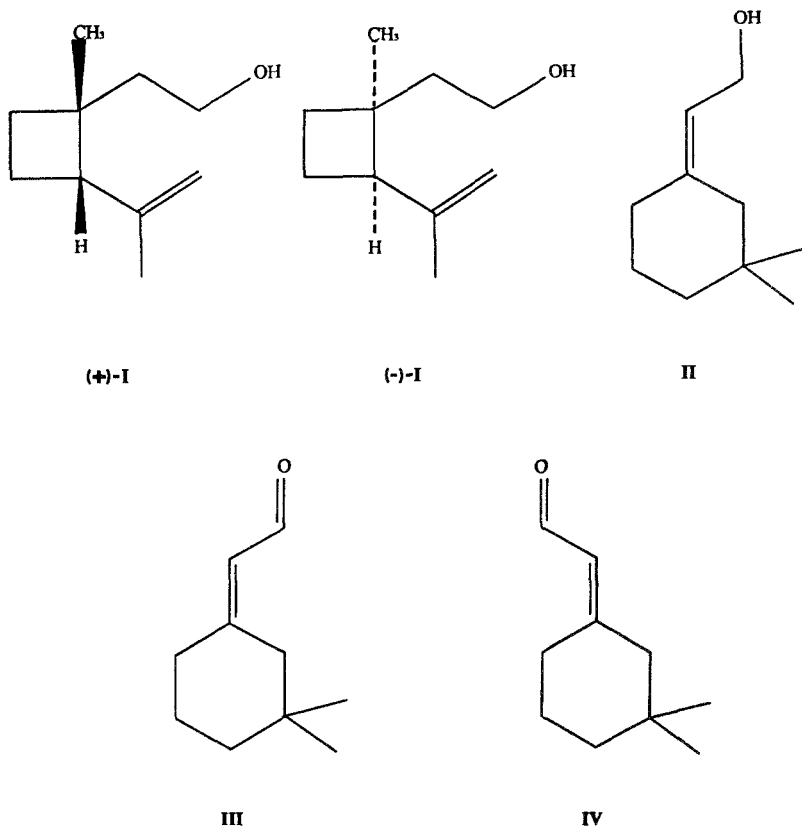


FIG. 1. Compounds composing the boll weevil aggregation pheromone (Tumlinson et al., 1969). Although (-)-I was not originally identified as a pheromone component, it is included since it was previously considered active (Mori et al., 1978) and occurs in current pheromone formulations as a result of racemic synthesis of I.

ies, groups of five insects of the same sex were maintained on moist filter paper in Petri dishes until use following receptor maturation at four days postemergence (Dickens and Moorman, 1987). Insects used in these studies were 4–7 days of age. All insects were held in incubators at 26°C under a photoregimen of 16 hr of light (ca. 700 lux) and 8 hr of darkness.

Chemicals. Racemic grandisol [= (±)-I], II, and III + IV (a 50:50 mixture as derived from a nonstereoselective synthesis), each >95% chemical purity, were obtained from Albany International, Controlled Release Division, Buckeye, Arizona. Compound III was 100% pure as prepared by the Mr. W.-C. Sun, State University of New York at Stony Brook (Prestwich et al., 1987)

by extensive recycle MPLC chromatography. Compound IV, used in electrophysiological studies, was >99% pure, while that used in the field studies had 96% geometrical purity. A 50:50 mixture of III (100% pure) and IV (>99% pure) was prepared for electrophysiological studies. Serial dilutions (0.0001–10 $\mu\text{g}/\mu\text{l}$) of all compounds were made in nanograde hexane. 1-Hexanol, used as a standard in electroantennogram (EAG) experiments, was 98% chemically pure as obtained from Aldrich Chemical Co., Milwaukee, Wisconsin.

Field Experiments. Compounds (\pm)-I, II, and the synthetic mixture of III + IV were apportioned as 10- μl aliquots of 10 $\mu\text{g}/\mu\text{l}$ dilutions. Compound III was apportioned as a 5- μl aliquot of a 10- $\mu\text{g}/\mu\text{l}$ dilution. Compound IV was apportioned as a 10- μl aliquot of a 5- $\mu\text{g}/\mu\text{l}$ dilution. (\pm)-I, and II, III, and IV were placed in separate 0.5-dram screw-cap vials using measured glass capillaries which remained in the vials. These vials were then sealed with Teflon-lined screw caps and placed in a cooler until use in the field. In the field, screw caps were removed from the vials, and both caps and vials were placed in the top of an aluminum film container (3 cm diam. \times 4.3 cm height) that had five holes (0.23 cm diam.) drilled near its outer edge. The bottom of the can was screwed onto the top, and the inverted container was then placed in the trap (modified after Mitchell et al., 1976; and in current use in the Boll Weevil Eradication Program) beneath the screen funnel.

The experimental design consisted of placing four traps in a square 20 m apart in an open, isolated area. The two treatments being tested were alternated among the traps. One hundred insects (50 males and 50 females) were released 20 m from each side of the square at ca. 1400 hr (400 insects total for each replicate of each experiment). Traps were then immediately baited. Each trap was checked, and the weevils removed on the day of release at 1500 hr, 1600 hr, and 1800 hr, and the following day at 1500 hr and 1600 hr, prior to the final trap check and removal of the baits at 0830 hr the next day.

In order to test the importance of each pheromone component, all possible three-component blends were each tested in competition with the four-component blend [(\pm)-I 50% (400 μg), II 37.5% (300 μg), and III 6.3% (ca. 50 μg) + IV 6.3% (ca. 50 μg) (100 μg)]. This blend of the four components was chosen since it approximated the natural mixture obtained from boll weevil frass: I, 52.3%; II, 39.4%; III, 4.1%; IV, 4.1% (Tumlinson et al., 1969), and relatively similar blends were significantly more attractive compared to other dissimilar blends (Hardee et al., 1974). The three-component blends tested in competition with the four-component blend were: (1) II (300 μg), III + IV (100 μg); (2) (\pm)-I (400 μg), III + IV (100 μg); (3) (\pm)-I (400 μg), II (300 μg), IV (50 μg); and (4) (\pm)-I (400 μg), II (300 μg), III (50 μg). The four-component blend was also tested against: (\pm)-I (400 μg), II (300 μg); and (\pm)-I (400 μg), II (300 μg), IV (100 μg). A competitive experiment tested the following three-component blends against each other: (\pm)-I (400 μg), II (300 μg), and III (50 μg) vs. (\pm)-I (400 μg), II (300 μg), and IV (50 μg). A control consisting of

(\pm)-I (400 μg), II (300 μg), and III + IV (100 μg) vs. hexane (80 μl) determined the attractiveness of the colored trap and solvent alone. Three replicates were run for each competitive treatment and the control.

Electrophysiology. EAGs were recorded as previously described (Dickens, 1984). Ag-AgCl capillary electrodes filled with physiological saline (Oakley and Schafer, 1978) were used. Following prepuncture with a sharpened tungsten needle, the recording electrode was inserted in the distal end of the club; the ground electrode was inserted into the distal end of the scape. The signal was amplified by a Grass P-16 DC preamplifier prior to visualization on a Tektronix 5111 analog oscilloscope and recording for storage with a strip-chart recorder.

Serial dilutions of III, IV, and a 50:50 mixture prepared from III and IV were delivered as 1- μl aliquots placed on filter paper (8 \times 18 mm) inserted into glass cartridges (80 mm \times 5 mm ID) oriented toward the preparation from a distance of 1 cm. Molecules evaporating from the filter paper were carried over the preparation by hydrocarbon-free air, which had been filtered and dried. Serial dilutions were delivered from the lowest to the highest concentration. Stimulus duration was ca. 1 sec with air flow of 1 liter/min. More than 3 min were allowed between presentations of olfactory stimuli, which was adequate for recovery of EAG activity.

Three replicates were recorded for serial dilutions of both III and IV, and a 50:50 mixture of III and IV for both male and female *A. grandis*. 1-Hexanol at the 100- μg dosage was used: (1) to normalize all responses; (2) to ensure viability and constancy of the preparation; and (3) to allow for relative comparisons with previously recorded species (Dickens, 1984; Dickens and Bolt, 1985; Dickens et al., 1986; Glancey and Dickens, 1988; Light et al., 1988). Stimulation with the standard both preceded and followed each serial dilution level. Responses to odorous stimuli at a given dosage were expressed as a percent of the mean of the two nearest responses to the standard (Dickens, 1981, 1984).

Statistical Analyses. Percent trap catches for simultaneous paired treatments in the field experiments were compared using a paired *t* test (Ostle, 1963). Results from tests not run simultaneously were compared by a *t* test for two means. Dosage-response curves resulting from the electrophysiological studies were compared by analysis of variance and Duncan's new multiple-range test (Duncan, 1955).

RESULTS

Three-Component Blends vs. Four-Component Blend. Competitive field experiments, in which each of the possible three-component blends were tested against the four-component mixture demonstrated the necessity of I, II, and IV

(Table 1). The removal of any one of these odorants led to a significant decrease in trap capture relative to the four-component blend ($P < 0.01$) with the attractancy of the resultant three-component mixtures not significantly different from the hexane solvent alone vs. the four-component blend. However, removal of III from the four-component mixture resulted in a three-component blend that did not differ significantly in trap capture from the four-component blend in competitive experiments.

Electrophysiological and Behavioral Experiments with III and IV. Since the (*Z*)-cyclohexylidene aldehyde, III, was not an essential component for capture of male and female boll weevils in traps in the field, we investigated further its detection and possible behavioral role in boll weevil aggregation. Dose-response curves constructed from EAGs recorded from male and female boll weevils to serial dilutions of III and IV showed both sexes to be 10–100 times more sensitive to IV (Figure 2). Antennal receptors of male boll weevils were significantly more responsive to IV than III ($P < 0.05$) at all concentrations after reaching threshold (ca. 0.1 μg) with an intermediate response to the mixture of geometric isomers (Figure 2a).

TABLE 1. MEAN PERCENT (\pm SD; $N = 3$) RESPONSE OF *Anthonomus grandis* IN COMPETITIVE FIELD TESTS WITH THREE-COMPONENT BLENDS VS. FOUR-COMPONENT BLEND, AND HEXANE SOLVENT VS. FOUR-COMPONENT BLEND^a

Treatment pair	$\bar{X}\%$ (\pm SD) capture/replicate	Sex ratio M:F
(\pm)-I, II, III + IV vs. II, III + IV	93.37** (\pm 7.21) 6.63 (\pm 7.21)	1.38:1 5.00:1
(\pm)-I, II, III + IV vs. (\pm)-I, III + IV	92.82** (\pm 5.09) 7.18 (\pm 5.09)	1.38:1 1.43:1
(\pm)-I, II, III + IV vs. (\pm)-I, II, IV	55.17 (\pm 6.21) 44.83 (\pm 6.21)	0.88:1 0.74:1
(\pm)-I, II, III + IV vs. (\pm)-I, II, III	86.93** (\pm 2.50) 13.07 (\pm 2.50)	0.94:1 1.00:1
(\pm)-I, II, III + IV vs. Hexane solvent	93.89** (\pm 4.44) 6.11 (\pm 4.44)	0.98:1 0.88:1

^a Dosages in each test were: (\pm)-I (400 μg), II (300 μg), III + IV (100 μg), III (50 μg), IV (50 μg), hexane solvent (80 μl).

** $P < 0.01$; paired *t* test.

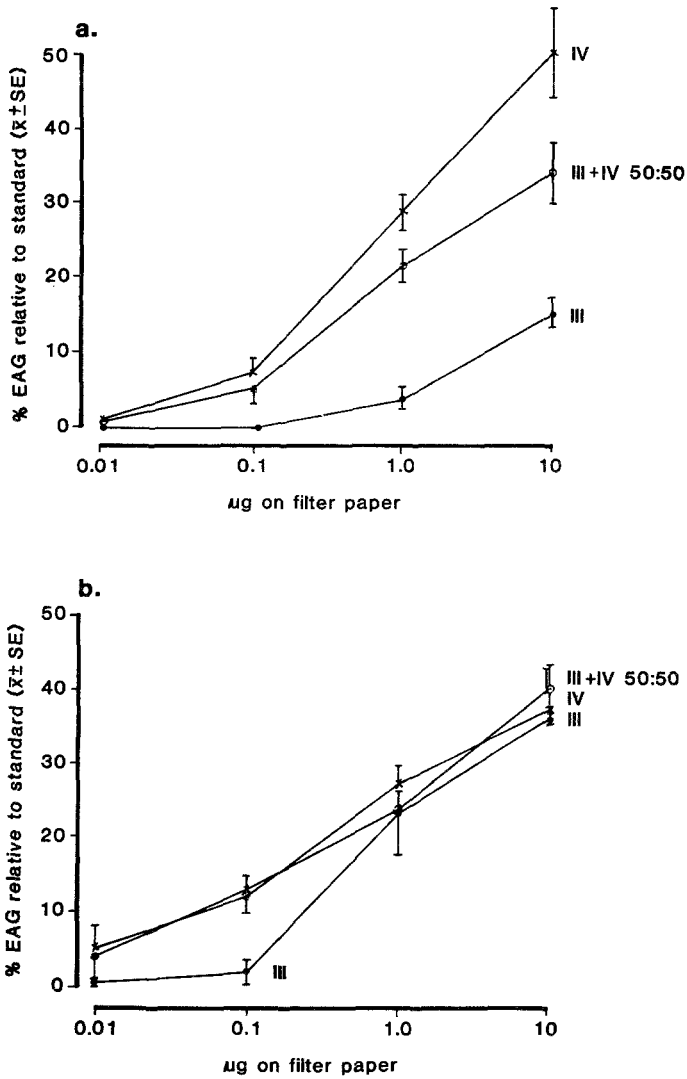


FIG. 2. Dosage-response curves constructed from EAGs of male (a) and female (b) *A. grandis* to serial dilutions of III, IV, and a 50:50 mixture of III and IV. Each point represents a mean of three replicates. Vertical bars represent standard errors.

Dosage-response curves for females to III, IV, and the mixture of III and IV differed from those obtained for males (Figure 2b). Responses of antennal receptors of females to serial dilutions of IV, and the III and IV mixture did not differ. Furthermore, after reaching threshold at 1 µg, responses to III did not differ from either IV alone or the mixture of III and IV.

Additional field experiments were conducted to determine: (1) whether IV was essential in the three-component blend of I, II, and IV when compared to the four-component blend; (2) whether increasing the dosage of IV affected trap capture by the three-component blend of I, II, and IV relative to the four-component blend; and (3) how the attractiveness of the three-component blend of I, II, and IV compared with the blend of I, II, and III (Table 2). The removal of IV decreased trap capture by the three-component blend of I, II, and IV when compared to the four-component blend (Table 2) to a level not significantly different from the other three-component blends or hexane control vs. the four-component blend (Table 1). Although doubling the amount of IV (100 μg) (Table 2) released with I and II in the three-component blend relative to our initial experiment (50 μg) (Table 1) against the four-component blend resulted in a higher percentage of weevils captured, this difference was not significant. In our final competitive field experiment, the blend of I, II, and IV captured significantly more weevils than I, II, and III ($P < 0.01$) (Table 2).

DISCUSSION

The essentiality of I, II, and IV for boll weevil aggregation is clearly demonstrated by our current competitive field tests with the three-component blends vs. the four-component blend. Previous knowledge of the activity of individual pheromone components and certain blends was based largely on results of lab-

TABLE 2. MEAN PERCENT (\pm SD; $N = 3$) RESPONSE OF *Anthonomus grandis* IN COMPETITIVE FIELD TESTS WITH FOUR-COMPONENT BLEND VS. I AND II; VS. I, II, IV (100 μg); AND I, II, IV VS. I, II, III^a

Treatment pair	$\bar{X}\%$ (\pm SD) capture/replicate	Sex ratio M:F
(\pm)-I, II, III + IV	93.49** (\pm 4.55)	1.04:1
vs.		
(\pm)-I, II	6.51 (\pm 4.55)	0.50:1
(\pm)-I, II, III + IV	42.78 (\pm 14.84)	0.77:1
vs.		
(\pm)-I, II, IV (100 μg)	57.24 (\pm 14.84)	1.20:1
(\pm)-I, II, IV	94.23** (\pm 3.68)	0.89:1
vs.		
(\pm)-I, II, III	5.77 (\pm 3.68)	0.67:1

^a Unless otherwise indicated, dosages in each test were: (\pm)-I (400 μg), II (300 μg), III + IV (100 μg), III (50 μg), IV (50 μg).

** $P < 0.01$; paired t test.

oratory bioassays done at the time of the initial identification of the pheromone (Tumlinson et al., 1969) and subsequent field tests (Hardee et al., 1974). While the laboratory bioassays showed various levels of activity for individual components and two-component blends, activity was substantially increased with the three-component blends of I, II, and IV, and especially I, II, and III, which was as attractive as live male boll weevils. The attractiveness of the four-component mixture exceeded both the live male boll weevils and each of the three-component blends (Tumlinson et al., 1969). However, the laboratory bioassay device used in these studies was an olfactometer in which the insects were offered a "choice" between various odorants and a blank or solvent control. These ambulatory responses of females to the various compounds and blends were then compared to female responses to live boll weevils in separate tests. Results from such an olfactometer can be misleading, since attraction may be related to the context in which an odorant or odorous blend is detected. Thus an odorant may elicit an attractive response when presented alone, but might be unattractive when presented in competition with another odorant, as previously shown for the boll weevil (Dickens, 1986). While previous field tests showed individual components to be unattractive, two- and three-component blends were not tested (Hardee et al., 1974). We point out that IV used in our current study was 96% chemically pure with 4% of its geometric isomer III. Thus we cannot yet rule out the possibility that III might be active as a minor or trace component of the attractant pheromone. This will await future experiments as additional quantities of III, and IV of greater purity become available. However, we can say that III (100% purity) alone is ineffective in enhancing trap capture in combination with I and II at the biologically meaningful dosages tested relative to the combination of IV with these odorants.

Both the low olfactory threshold for IV, and the significantly lower EAGs elicited by III relative to IV for male boll weevils (Figure 2) correlate well with the role of IV as a pheromone component. The dosage-response curve for III was parallel to that of IV, only shifted closer to the abscissa. A dosage-response curve intermediate and parallel to the other two was obtained for the mixture of III and IV. Similar results were obtained for the enantiomers of grandisol (I) in the boll weevil (Dickens and Mori, 1989) and are consistent with a single acceptor type for IV (Kaissling, 1971). However, while antennal olfactory receptors of females were more sensitive to IV than III, EAGs elicited at higher dosages by III, IV, and a mixture of III and IV were not significantly different (Figure 2). Furthermore, shapes of dosage-response curves for III and IV for females were different. Dosage-response curves of different shapes, and responses to the III and IV mixture which were similar to III and IV alone at higher dosages, may be indicative of separate populations of acceptors for III and IV in females. Detection of III by female boll weevils plays no apparent role in their orientation to pheromone traps in the current study, since sex ratios

seemed to vary among tests rather than individual treatments within a test (Tables 1 and 2). It might be speculated that III may function in close range orientation of females to pheromone-producing males and/or in the assessment of oviposition sites by providing a cue to avoid squares already damaged by male feeding.

If III is not essential in the boll weevil attractant response, then what is its function? As mentioned before, III might play a role in short-range orientation and/or other behaviors of females. Compound III might also be involved in the biosynthesis of II through a stereoselective aldehyde reductase or some other biosynthetic mechanism. It is also possible that the (*Z*)-cyclohexylidene aldehyde, III, arises by nonbiological oxidation of the (*Z*)-cyclohexylidene allylic alcohol II or that III is intermediate in the degradation of the alcohol II to an inactive carboxylic acid (Sun and Prestwich, unpublished). In this regard, both the biosynthesis of lepidopterous pheromones (Morse and Meighen, 1986) and the conversion by microsymbionts of certain host monoterpenes to bark beetle pheromones (Brand et al., 1975; Brand, 1976; Leufven et al., 1986) involve alcohol precursors that are oxidized to aldehydes or ketones. The possible role of III in pheromone biosynthesis or degradation (Prestwich, 1987) and in boll weevil behavior will be investigated further.

Our discovery of the relative inactivity of III in boll weevil trap capture, and our recent elucidation of the lack of detection and behavioral response of boll weevils to (-)-I (Dickens and Mori, 1989) could be of importance in current boll weevil eradication/suppression programs (Ridgway and Lloyd, 1983). These programs depend heavily on traps baited with (\pm)-I, II, III, and IV for detection of boll weevils for both survey and evaluation of various control measures. When one considers the inactivity of (-)-I and III, then inactive compounds make up 40% of the current bait. Furthermore, increased ratios of III + IV relative to I and II decrease the attractiveness of the four-component mixture (Hardee et al., 1974; Huddleston et al., 1977), and (-)-I might be slightly inhibitory (Dickens and Mori, 1989). In light of our current knowledge, the effects of increasing III + IV on trap capture may be due to III alone, since increasing IV in our experiments only led to increased trap captures (Tables 1 and 2). We are currently investigating the possibility of obtaining I composed primarily of the (+)-enantiomer and IV separated from III for more efficient use of the pheromone in field programs.

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VARIATION OF ENANTIOMERIC COMPOSITION OF α -PINENE IN NORWAY SPRUCE, *Picea abies*, AND ITS INFLUENCE ON PRODUCTION OF VERBENOL ISOMERS BY *Ips typographus*¹ IN THE FIELD²

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Abstract—The enantiomeric composition of α -pinene in individual Norway spruce trees [*Picea abies* (L.) Karst.] was determined on a chiral GC column after stereoselective hydroboration–oxidation followed by a reaction with isopropyl isocyanate to form the carbamate derivative. The enantiomeric composition varied considerably between trees of different genetic origin. There was a strong correlation between the chirality of α -pinene in host spruce trees and the *cis/trans* ratio of verbenols found in the hindguts of the bark beetle *Ips typographus* (L.) infesting the trees.

Key Words—Enantiomeric composition, chiral separation, GC, pheromone, plant–insect relations, Norway spruce, *Picea abies*, α -pinene enantiomers, *cis*-verbenol, *trans*-verbenol, *Ips. typographus*, Coleoptera, Scolytidae.

INTRODUCTION

Many bark beetles in the genus *Ips* are known to produce *cis*- and *trans*-verbenol when they are exposed to the vapors of α -pinene or oleoresins from their

¹Coleoptera; Scolytidae.

²Parts of this work have been presented at the 14th International Symposium on the Chemistry of Natural Products in Poznan, Poland, July 9–14, 1984.

host trees (Renwick et al., 1976; Byers, 1981). This production, which is a stereospecific allylic hydroxylation, gives (1*S*, 4*S*, 5*S*)-*cis*-verbenol from (–)-1*S*, 5*S*)- α -pinene, whereas (1*R*, 4*S*, 5*S*)-*trans*-verbenol is produced from the (+)-(1*R*, 5*R*)-enantiomer of α -pinene. Moreover, males of *I. typographus* produce *cis*- and *trans*-verbenol in ratios that were found to reflect the enantiomeric composition of the α -pinene, to which they were exposed in a laboratory experiment (Klimetzek and Francke, 1980). (*S*)-*cis*-Verbenol is also identified as a component of the aggregation pheromones of both *I. paraconfusus* (Silverstein et al., 1966) and *I. typographus* (Krawielitzki et al., 1977), while the attraction to *trans*-verbenol is small or uncertain (Dickens, 1981; Schlyter et al., 1987).

It is well known that there are genetic and seasonal variations of the monoterpene compositions of conifers (Juvonen, 1966; Rudloff, 1967). However, very little is known about the enantiomeric compositions due to the lack of convenient analytical methods.

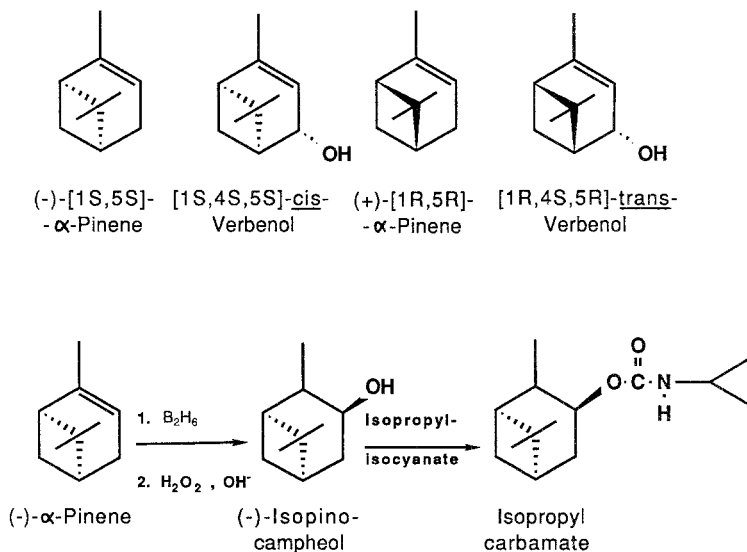
α -Pinene is produced in different enantiomeric compositions by various conifer species (Klimetzek and Francke, 1980). Even within the same genus, i.e., *Pinus*, different species are known to vary from almost pure (+)- to almost pure (–)-enantiomer; α -pinene from *P. halipensis* exhibits $[\alpha]_D = +48.30^\circ$, whereas that of *P. pinaster* (French turpentine) has $[\alpha]_D = -43.4^\circ$ (Mirov, 1961). Pure (+)- α -pinene has been reported to have $[\alpha]_D = +52.4^\circ$ (Comyns and Lucas, 1957). The intraspecific variation in the enantiomeric composition of monoterpene hydrocarbons, such as α -pinene, has to our knowledge not been investigated.

The methods available for the determination of the enantiomeric compositions of hydrocarbons are limited. The classical polarimetric method and NMR techniques require comparatively large amounts of pure samples and are not suitable for complex mixtures, whereas chromatographic techniques require relatively small sample amounts, and crude samples of complex mixtures can be analyzed.

Several chiral GC phases are known, and it is also possible to separate enantiomers as diastereomers on ordinary GC phases, but none of these techniques is general or works well for the resolution of monoterpene hydrocarbon enantiomers. The aim of the present investigation was to develop a sensitive chromatographic method for the determination of the enantiomeric composition of α -pinene in biological samples. Recently, methods for gas-chromatographic determinations of the enantiomeric compositions of secondary alcohols have been reported. The optical purity can be determined either via separation of diastereomeric derivatives [N-TFA-(L)-alanine esters] (Kruse et al., 1979) or by separation of isopropyl carbamates on a chiral GC phase, Chirasil-Val (König et al., 1982). There are also some reports on direct separation of alcohol enantiomers using chiral metal complexes as GC phase (Schurig et al., 1985).

It is possible to convert olefinic hydrocarbons regio- and stereoselectively

into secondary alcohols via a hydroboration–oxidation reaction (Zweifel and Brown, 1964). We have applied this reaction to convert α -pinene into isopinocampheol. For this purpose we have used the two-phase technique of Brändström et al. (1972). The isopinocampheol was separated as its isopropyl carbamate according to the method of König et al. (1982) (Scheme 1).



SCHEME 1.

We have used this method to study the enantiomeric composition of α -pinene in individual spruce trees, *Picea abies*, and compared it to the *cis/trans*-isomer ratio of verbenol found in the hindguts of male bark beetles, *Ips typographus*, attacking some of these trees.

METHODS AND MATERIALS

Biological Material. Phloem samples were taken from 29 different individual spruce trees [*Picea abies* (L) Karst.] at three different localities. The first was a forest plantation close to Uppsala, 70 km north of Stockholm. Samples were taken from 21 different 10-year-old cloned trees originating from Finland (SF) and West Germany (BRD). These samples were taken in February 1983 and stored as logs for six to eight months in individual polyethene bags at -18°C . Two spruce trees close to the laboratory in Stockholm made up a second group. The third source of phloem samples were six trees infested by spruce

bark beetles, *Ips typographus*, sampled close to Torsby, province of Värmland, western Sweden, during the main swarming period in early June 1982 (cf. Birgersson et al., 1988). These phloem samples were removed with a knife and stored in liquid nitrogen until extraction and analysis.

From each spruce tree in the third group, male bark beetles were also collected and analyzed for their hindgut volatiles as described in detail by Birgersson et al. (1984).

Sample Preparation. The outer cork bark was removed with a knife, and the phloem sample was removed from the log with a rasp. On the infested trees the stored phloem sample was chopped with a knife. The phloem sample (1 g) was extracted with pentane (Merck p.a.) for 15 min in an ultrasonic bath. The pentane extract was washed with saturated sodium bicarbonate and passed through a short alumina column (2 g Merck, Aktiv 60, base treated) to remove chlorophyll and polar compounds. Most of the pentane was evaporated and dry dichloromethane (1 ml) was added. The solution was transferred to a 3-ml reaction vial equipped with a PTFE-coated septum. Tetrabutylammonium boronate (Brändström et al., 1972) (50 mg) was added. The vial was flushed with argon. Ethyl bromide (50 μ l) was added with a syringe, and the reaction mixture was kept at 60°C for 30 min. A few drops of water were added to destroy the excess of hydride. Aqueous sodium hydroxide (2 N, 100 μ l) was added, followed by a slow addition of hydrogen peroxide (35%, 70 μ l). The vial was left at room temperature for 1 hr. Most of the dichloromethane was evaporated, and water and pentane were added. The pentane phase was separated and concentrated.

The concentrated pentane extract was dissolved in dichloromethane (200 μ l), and isopropyl isocyanate (100 μ l) was added. The mixture was heated to 100°C for 20 min. Dichloromethane and the excess of isopropyl isocyanate were evaporated and the residue dissolved in pentane and analyzed on GC.

The gas chromatographic analyses were performed on a PYE 204 GC and on a Hewlett-Packard 5830 GC both equipped with fused silica columns coated with XE-60-(S)-valine-(S)- α -phenylethylamide (Chrompac 50 m, ID 0.23, $df = 0.14$ mm, HETP = 0.21 mm). The oven was kept at 165°C isothermal, and injector and detector temperatures were 250°C. Helium was used as carrier gas (16–22 cm/sec). The α -factor for the two enantiomers was 1.0178.

Preparation of Test Mixtures and Isopinocampheol References. A test sample containing 60.0% of (–)- α -pinene was prepared by mixing (+)- and (–)- α -pinene isolated earlier at the Department of Organic Chemistry, Royal Institute of Technology (0.045 g of $[\alpha]_D = 19.6^\circ$ and 0.053 g of $[\alpha]_D = -36^\circ$) and used to check the steric course of the hydroboration step. Small samples (25, 50, 100, and 150 μ g) were taken from the test mixture and hydroborated as described for the pentane extracts of spruce trees.

Isopinocampheol. Ethyl bromide (3.27 g) was slowly added to a solution of tetrabutylammonium boronate (2.57 g) and α -pinene (2.72 g) of high optical

purity (Fluka) in dry dichloromethane (10 ml) under an atmosphere of argon. The mixture was refluxed for 30 min. The excess of hydride was destroyed with water. Aqueous sodium hydroxide (2 N, 5 ml) was added, followed by dropwise addition of hydrogen peroxide (35%, 3 ml). The mixture was stirred at room temperature for 1 hr. The dichloromethane phase was separated and dried, and the solvent was slowly evaporated to yield crude isopinocampheol, which was used as reference compound for the GC analysis.

RESULTS AND DISCUSSION

The α -pinene samples from the test mixture were analyzed and found to contain $60.9 \pm 0.7\%$ (min 59.9%, max 61.4%, $N = 6$) of the (-)-enantiomer, close to the expected 60.0%. The experiment shows that the hydroboration is stereoselective and that both accuracy and precision of this method for detecting the enantiomeric composition of α -pinene are good.

The enantiomeric composition of α -pinene in the phloem of Norway spruce trees was found to vary between 32 and 98% of the (-)-enantiomer (corresponding to 38% ee of (+)- α -pinene and 96% ee of (-)- α -pinene) (Table 1).

Males of the spruce bark beetle (*Ips typographus*) were collected from six infested Norway spruce trees and the hindguts were extracted and analyzed for the total amount of *cis*-verbenol (Birgersson et al., 1988) and the ratio of *cis*- to *trans*-verbenol. The relative amounts of verbenol isomers correlated strongly ($r = 0.91$, $P < 2\%$) to the relative amounts of (-)- and (+)- α -pinene in the phloem (Figure 1). This result is in accordance with the laboratory experiments performed by Klimetzek and Francke (1980).

TABLE 1. PERCENTAGE OF (+)-ENANTIOMER OF TOTAL AMOUNT OF α -PINENE IN NORWAY SPRUCE

	N^a	$\bar{X} \pm \text{S.D.}$	Min	Max
Trees BRD ^b	11	12.9 \pm 9.9	1.9	30.0
Trees SF ^c	10	36.9 \pm 11.2	15.0	51.3
Infested	6	31.3 \pm 23.0	5.0	61.0
Trees Sthlm ^d	2		13.0	67.9
All trees	29	27.5 \pm 18.9	1.9	67.9

^aNumbers of trees investigated.

^bTrees originating from three different clones from W. Germany.

^cTrees originating from three different clones from Finland

^dTrees from Stockholm.

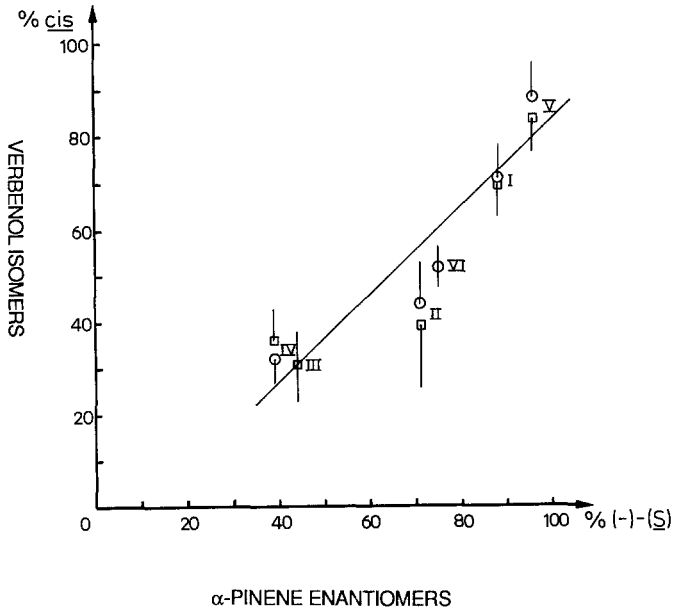


FIG. 1. Percentage *cis* isomer of verbenol from *Ips typographus* male hindguts versus percentage (-)-enantiomer of α -pinene found in *Picea abies* phloem extracts from infested trees (I–VI). \square = attack phase 3, $\bar{X} \pm \text{SD}$; \circ = attack phase 6, $\bar{X} \pm \text{SD}$, attack phases according to Birgersson et al. (1984). ($N = 10$; $k = 0.91$; $m = 9.23$ ($y = kx + m$); $r = 0.91$; $r^2 = 0.82$.) In tree III, no beetles in attack phase 6 were found, and in tree VI no beetles in attack phase 3 were found.

The intraspecific variation in the enantiomeric composition of α -pinene in spruce was probably the reason for the findings by Birgersson et al. (1984) that batch samples of *I. typographus* males attacking different spruce trees varied in their ratios of *cis*- and *trans*-verbenols.

Since *cis*-verbenol is an essential component of the aggregation pheromone of *Ips typographus* and as there is a direct relation between the *cis* ratio of verbenols found in the hindguts of the bark beetle and the enantiomeric composition of α -pinene in the phloem of the host tree, we assume that there also exists a relationship between the enantiomeric composition in the Norway spruce and its resistance to attacks of *Ips typographus*. Trees with a very low content of (-)- α -pinene may thus be resistant to bark beetle attacks. Further studies to test this hypothesis are in progress.

There is also a strong indication that the enantiomeric composition within a clone is constant. However, the amount of material investigated is too small

to settle this point. Work is in progress to study the inheritance of the enantiomeric composition of α -pinene.

During the course of this investigation, a new method using α -cyclodextrin and formamide as the stationary phase in gas chromatography for the separation of α -pinene has been published (Koscielski et al., 1983). This method has been shown to be very useful for the enantiomeric separation of several of the common monoterpene hydrocarbons (Lindström et al., 1987). However, further development is needed before this method can be applied to complex mixtures.

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INTERSPECIFIC VARIATION IN DEFENSE SECRETIONS OF MALAYSIAN TERMITES FROM THE GENUS *Nasutitermes* (ISOPTERA, NASUTITERMITINAE)

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Abstract—The defense secretions of five species of Malaysian *Nasutitermes*, *N. longinasus*, *N. matangensis*, *N. havilandi*, *N. johoricus*, and *Nasutitermes species 01*, are compared. *N. longinasus* and *N. species 01* provide triterpene alcohols, *N. havilandi* mainly tricyclic trinervitene and tetracyclic kempene alcohols and acetates, whereas *N. matangensis* furnish acetyl/propionyl derivatives of trinervita-11(12),15(17)-dien-3 α ,9 β ,13 α -triol (XXI and XXII). A new diterpene, assigned as trinervita-11(12),15(17)-dien-3 α ,13 α -diol-3,13-*O*-diacetate (XVII), is isolated from *N. havilandi*. The mono- and diterpenoid compositions, being species-specific, are useful for chemotaxonomic and phylogenetic studies.

Key Words—Isoptera, Termitidae, defense secretions, interspecific variations, *Nasutitermes*, diterpenes.

INTRODUCTION

The genus *Nasutitermes* belongs to the subfamily Nasutitermitinae in which the soldiers are characterized by conical, nozzle-shaped projections from which they eject defensive secretions produced by the enlarged frontal glands located within the head capsules. Behaviorally, most *Nasutitermes* species forage under covered trails. The covered trails may run extensively on the jungle floor and up along tree trunks, with most species feeding on dead plant material. The soldiers protect their foraging trails by positioning themselves along the fringes once

the trail is disturbed or exposed. Defense is effected by ejecting gluey secretions at their enemies. Although Malaysian *Nasutitermes* are very widespread, they are less studied than the free-ranging genera such as *Laccositermes*, *Hospitalitermes*, and *Longipeditermes* (Goh et al., 1984). The biology of Malaysian *Nasutitermes* is still relatively unknown. This paper presents the compositional and structural variations of the defense secretions of the soldier termites of *N. longinasus*, *N. matangensis*, *N. havilandi*, *N. johoricus*, and an unidentified species which will be referred to as *N. species 01*.

METHODS AND MATERIALS

Termite Material. Soldier termites used for the present studies were all collected from within closed canopy dipterocarp forests from the following localities of Peninsular Malaysia (Figure 1): (1) Gunong Jerai forest reserve [5°40'N, 100°15'E; 1200 m above sea level (ASL)]—an upper hill dipterocarp forest in the state Kedah; (2) Sungei Lallang forest reserve (5°30'N, 100°20'E; 50 m ASL)—a logging forest in the state of Kedah; (3) Muka Head Field Station (5°20'N, 100°8'E; 200 m ASL)—a coastal hill dipterocarp forest reserve in Penang; (4) Taman Negara (4°20'N, 102°25'E; 1,000 m ASL)—the national park of Peninsular Malaysia in the state of Pahang; (5) Jerantut forest reserve (3°50'N, 102°20'E; 50 m ASL)—a forest reserve area in the states of Pahang, Trengganu and Kelantan; (6) Cameron Highlands (4°30'N, 101°25'E; 1500 m ASL)—a highland resort in the state of Pahang; (7A) Gombak forest reserve (3°20'N, 101°46'E; 300 m ASL); (7B) Genting Sempah forest reserve (3°14'N, 101°46'E; 500 m ASL); (7C) Templer Park (3°22'N, 101°40'E; 200 m ASL); (7D) Gunong Sempit forest reserve (3°20'N, 100°49'E; 500 m ASL); (7E) Puchong forest reserve (3°10'N, 100°40'E; 50 m ASL)—7A–7E are hill dipterocarp forests in the state of Selangor; (8) University of Malaya Campus (3°10'N, 100°40'E; 50 m ASL)—a hill forest reserve at university campus in Wilayah Persekutuan; and (9) Pasoh forest reserve (2°58'N, 101°55'E; 100 m ASL)—a lowland dipterocarp forest in the state of Negeri Sembilan which has been the site for intensive ecological studies under the auspices of the International Biological Programme.

Analytical Methods. Gas chromatography (GC) was performed on a HP 5790a instrument fitted with a flame ionization detector. Two glass columns (1.6 m × 3 mm 3% OV-17 and 1.6 m × 3 mm 3% OV-101 on 100–120 Gas Chrom Q) were used for the diterpenoids and a 3-m × 5-mm stainless-steel column packed with 10% Carbowax 20 M on 120–140 Gas Chromsorb Q was used for the monoterpenes. Gas chromatography–mass spectrometry (GC-MS) was performed on a Pye 104 gas chromatograph interfaced to a double-beam Kratos MS30 mass spectrometer by a membrane separator. Mass spectra were

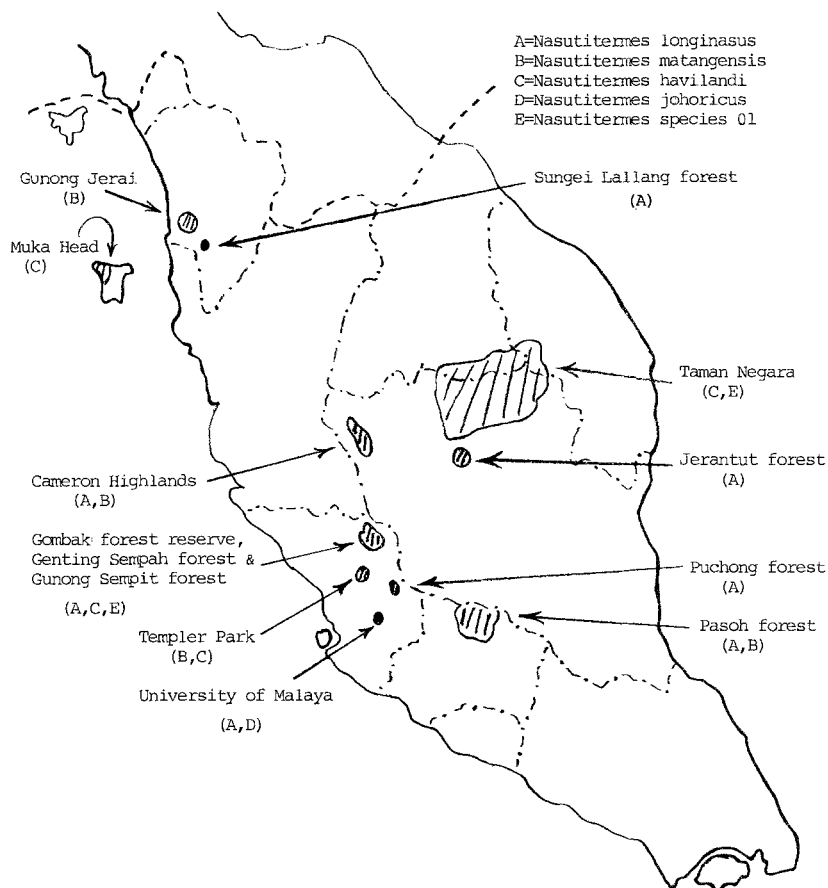


FIG. 1. Distribution of *Nasutitermes* in peninsular Malaysia.

obtained at 70 eV with the MS source at 100°C and membrane interface temperature at 230°C. Mass spectral data were accumulated by a Kratos DS55 data system. The data on the monoterpenes, characterized by GC, GC-MS and coinjection with standards, are summarized in Table 1. The diterpenes (VI-XXII) were characterized by the NMR and MS spectral comparison to reported data (Vrkoc et al., 1978a,b; Prestwich, 1979a,b; Dupont et al., 1981) and by coinjection with compounds previously identified (Chuah et al., 1983, 1986; Goh et al., 1982, 1984). The results are summarized in Table 2. The entire gas chromatographic profile of each defense secretion was typically obtained by on-column injection at 90°C and then programmed at 8°C/min to 300°C on 1.6 m × 3-mm 3% OV-101 and 1.6-m × 3-mm 3%-OV-17 glass

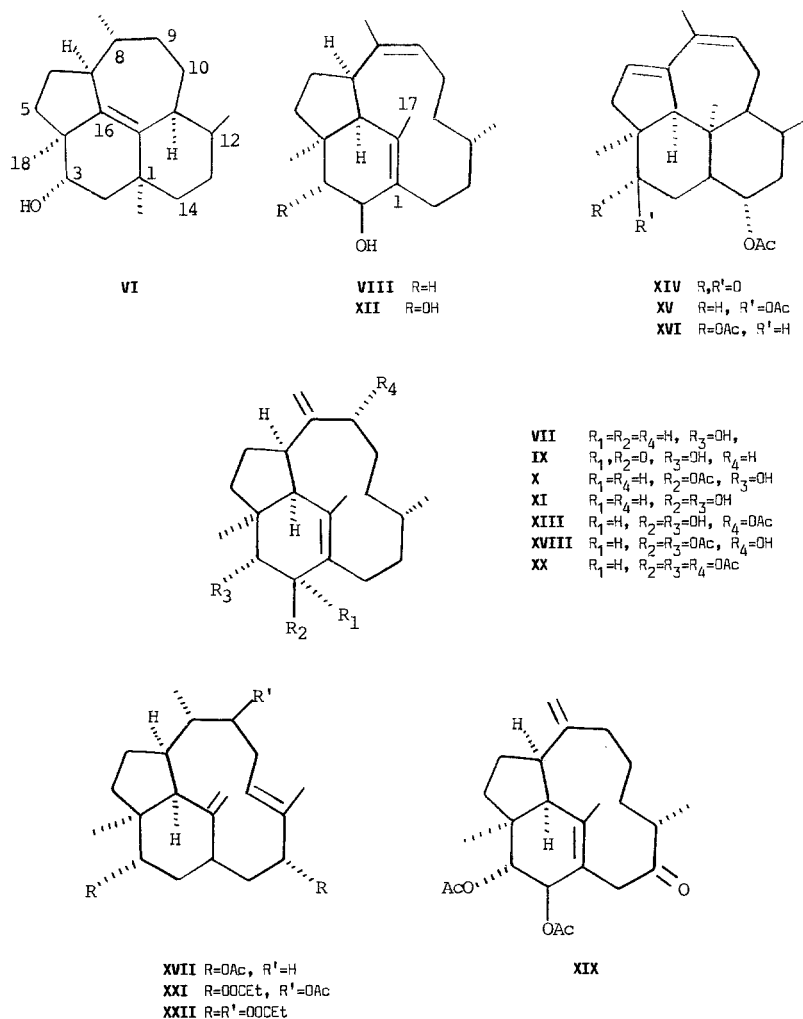


FIG. 2. Diterpenes from the defense secretions of Malaysian *Nasutitermes*.

columns. Retention indices of all diterpenes were made relative to n-alkane standards, and R_f values on silica gel TLC plates are summarized in Table 3 below.

Small-scale preparative separations were by HPLC on a Waters 440 instrument fitted with a 25-cm \times 1/4-in Ultrasphere silica gel column. Elution of the diterpenoids was by a gradient of 5–20% ethyl acetate in hexane. All isolated diterpenes were checked by GC and TLC on 5 \times 10-cm 0.25-mm silica gel 60

TABLE I. INTERSPECIFIC VARIATION OF MONOTERPENE HYDROCARBON COMPOSITION (%) IN *Nasutitermes*

Compounds	Termites					
	<i>N. longinasus</i> ^a		<i>N. havilandi</i> ^b	<i>N. johoricus</i> ^c	<i>N. matangensis</i> ^d	<i>N. species 01</i> ^e
	Minor	Major				
α -Pinene (I)	94-98	95-98	70-80	98-99		
β -Pinene (II)	1-5	1-4	18-24	1-2		
Myrcene (III)					73-81	
Limonene (IV)	1-2	0-1	1-7		19-27	1-2
Terpinolene (V)						98-99

^aRange for 16 colonies.

^bRange for 8 colonies.

^cRange for 2 colonies.

^dRange for 8 colonies.

^eRange for 3 colonies.

plates. The isolated known diterpenes were identified by their [¹H]NMR and mass spectral data and by comparison of their *R_f* and retention times with those previously identified (Chuah et al., 1983, 1986; Vrkoc et al., 1978a,b; Prestwich, 1979a,b; Dupont et al., 1981). In *N. havilandi*, a new diterpene (XVII) was obtained and, based on NMR, IR and MS data, its structure was assigned as trinervita-11(12),15(17)-dien-3 α ,13 α -diol-3,13-*O*-diacetate (XVII). The mass spectrum of this diterpene, XVII, showed peaks at *m/z* 388 (M^+ , 1, $C_{24}H_{36}O_4^+$), 328 (5, $M^+ - AcOH$), 286 (4, $M^+ - AcOH - CH_2CO$), 268 (100%, $M^+ - 2AcOH$), 253 (26, $M^+ - 2AcOH - CH_3$), 135 (27, $C_{10}H_{15}^+$), 60 (30, $AcOH^+$), 43 (72, CH_3CO^+), 41 (15, $C_3H_5^+$). Calculated exact mass for $C_{24}H_{36}O_4$, 388.2613; found, 388.2508. [¹H]NMR [100 MHz] and [¹³C]NMR [25 MHz] spectra are given below in Tables 5 and 6, respectively.

RESULTS AND DISCUSSION

The evolution and development of the frontal gland in the subfamily Nasutitermitinae, of which over 500 species have been reported (Tho, 1982), must be closely paralleled by the development of chemicals for the defense of the termite colony against predators. The biosynthesis of such chemicals in the cephalic gland has provided the termites with a vast array of defense chemicals against attacks by hostile organisms with whom they have to share their environment.

TABLE 2. INTERSPECIFIC VARIATION OF DITERPENE COMPOSITION (%) IN *Nasutitermes*

Compounds	Termites					
	<i>N. longinasus</i> ^a		<i>N. havilandi</i> ^b	<i>N. johoricus</i> ^c	<i>N. matangensis</i> ^d	<i>N. species OI</i> ^e
	Minor	Major				
15-Ripperten-3 α -ol (VI)	1-4	1-5				1-2
Trinervita-1(15),8(19)-dien-3 α -ol (VII)	5-8	3-8				2-4
Trinervita-1(15),8(9)-dien-3 α -ol (VIII)	3-7	3-8				3-5
2-Oxotrinerivita-1(15),8(19)-dien-3 α -ol (IX)	4-14	4-10	0-1			
Trinervita-1(15),8(19)-dien-2 β ,3 α -diol-2- <i>O</i> -acetate (X)	1-6	2-4				2-10
Trinervita-1(15),8(19)-dien-2 β ,3 α -diol (XI)	31-45	20-29	2-3			40-52
Trinervita-1(15),8(9)-dien-2 β ,3 α -diol (XII)	15-22	26-40	0-1			19-25
Trinervita-1(15),8(19)-dien-2 β ,3 α ,9 α -triol-9- <i>O</i> -acetate (XIII)	10-19	8-21	1-2			8-15
3-Oxokempa-6,8-dien-14 α -ol-14- <i>O</i> -acetate (XIV)			38-46			
Kempa-6,8-dien-3 β ,14 α -diol-3,14- <i>O</i> -diacetate (XV)			13-21			
Kempa-6,8-dien-3 α ,14 α -diol-3,14- <i>O</i> -diacetate (XVI)			20-26			
Trinervita-1(12),15(17)-dien-3 α ,13 α -diol-3,13- <i>O</i> -diacetate (XVII)			3-10			

Trinervita-1(15),8(19)-dien-2 β ,3 α ,9 α -triol-2,3- <i>O</i> -diacetate (XVIII)	2-6	1-6	2-5	
13-Oxotrinervita-1(15),8(19)-dien-2 β ,3 α -diol-2,3- <i>O</i> -diacetate (XIX)			1-3	
Trinervita-1(15),8(19)-dien-2 β ,3 α ,9 α -triol-2,3,9- <i>O</i> -triacetate (XX)	1-3	1-2		
Trinervita-11(12),15(17)-dien-3 α ,9 β ,13 α -triol-9- <i>O</i> -acetyl-1,13-dipropionate (XXI)				12-21
Trinervita-11(12),15(17)-dien-3 α ,9 β ,13 α -triol-3,9,13- <i>O</i> -tripropionate (XXII)				77-88
Unknown U1				20-22
Unknown U2				78-80

^aRange for 16 colonies.

^bRange for 8 colonies.

^cRange for 2 colonies.

^dRange for 8 colonies.

^eRange for 3 colonies.

The results from the present study of the defense secretions of five *Nasutitermes* species show that there are marked differences in the distribution of monoterpenes (Table 1) and diterpenes (Table 2). Interspecifically, these species can be differentiated by such variations, including the presence or absence of certain diterpenes, which generally have complicated structures or display complex compositional patterns. Intraspecifically, there are only small but detectable quantitative variations in monoterpene and diterpene composition. The monoterpene components in the defense secretions of *N. longinasus* and *N. havilandi* comprise α -pinene, β -pinene and limonene, whereas in *N. matangensis* the monoterpenes are predominantly myrcene and limonene. The monoterpene contents in *N. johoricus* comprise α - and β -pinenes, whereas in *N. species 01* predominant amounts of limonene and terpinolene are shown. The diterpenes of these five species also show marked interspecific variations. In this respect, *N. longinasus* produces mainly the common diterpene mono-alcohols VI, VII, and VIII; diols XI and XII; diol monoacetate X; and triol monoacetate XIII; whereas the *N. havilandi* soldier secretes a mixture of ketokempene monoacetate XIV, tricyclic diterpene diacetate XVII, and kempene diacetates XV and XVI. In *N. matangensis*, the soldier secretes the dipropionate monoacetate XXI and the tripropionate XXII, whereas *N. johoricus* secretes two unknown diterpenes U1 and U2, which are different from those given above as indicated by GC and TLC data (Table 3). The occurrence of the above propionate esters (XXI and XXII) reported earlier (Prestwich et al., 1981c) and now confirmed as secreted from the species *N. matangensis*, provides clues to the biogenetic pathway of the various diterpene structures. In this respect, the presence of the 11(12)-olefinic bonds in the tricyclic skeleton (e.g., XXI and XXII) allows for the biosynthetic ring closure to tetracyclic kempene (XV) and rippertane (VI) as well as spirotetracyclic longipane structures (Goh et al., 1984).

The chemistry of the secretion of *N. species 01* is quite similar to that of *N. longinasus* but can be differentiated on the basis of monoterpene distributions (Table 1). In the former species, the soldiers are monomorphic, while those of *N. longinasus* are dimorphic. Heads of the major soldiers of *N. longinasus* are relatively bigger than the heads of *N. havilandi*, *N. johoricus*, and *N. matangensis*; thus the amount of its defense secretion is relatively higher (Table 4). As with many other genera, the size of the soldier head gives an indication of the storage capacity of the defense secretion.

Four major compounds were isolated from extracts of *N. havilandi*. Three compounds, viz., kempa-6,8-dien-3 β ,14 α -diol-3,14-*O*-diacetate (XV), kempa-6,8-dien-3 α ,14 α -diol-3,14-*O*-diacetate (XVI), and 3-oxokempa-6,8-dien-14 α -ol-14-*O*-monoacetate (XIV), are similar to those reported for another Malaysian termite *Bulbitermes singaporensis* (Prestwich et al., 1981b). The mass spectrum of the new diterpene diacetate XVII with M^+ at 388, compatible with

TABLE 3. CHROMATOGRAPHIC PROPERTIES OF DITERPENES FROM *Nasutitermes*

Compound	Mol wt.	R_f (color) ^a	OV-1 ^b	OV-17 ^c
15-Ripperten-3 α -ol (VI)	288	0.48 (sky blue)	21.8	24.1
Trinervita-1(15),8(19)-dien-3 α -ol (VII)	288	0.58 (wisteria violet)	22.0	24.3
Trinervita-1(15),8(9)-dien-3 α -ol (VIII)	288	0.54 (violet)	22.3	24.7
2-Oxotrinervita-1(15),8(19)-dien-3 α -ol (IX)	302	0.61 (lilac)	22.8	25.5
Trinervita-1(15),8(19)-dien-2 β ,3 α -diol-2- <i>O</i> -acetate (X)	346	0.36 (pink)	23.5	26.2
Trinervita-1(15),8(19)-dien-2 β ,3 α -diol (XI)	304	0.23 (pink)	23.5	26.2
Trinervita-1(15),8(9)-dien-2 β ,3 α -diol (XII)	304	0.16 (pink)	24.0	26.7
Trinervita-1(15),8(19)-dien-2 β ,3 α ,9 α -triol-9- <i>O</i> -acetate (XIII)	362	0.14 (deep purple)	24.5	27.0
3-Oxokempa-6,8-dien-14 α -ol-14- <i>O</i> -acetate (XIV)	342	0.58 (green)	24.9	27.2
Kempa-6,8-dien-3 β ,14 α -diol-3,14- <i>O</i> -diacetate (XV)	386	0.58 (green)	25.5	28.0
Kempa-6,8-dien-3 α ,14 α -diol-3,14- <i>O</i> -diacetate (XVI)	386	0.57 (deep purple)	25.9	28.0
Trinervita-11(12),15(17)-dien-3 α ,13 α -diol-3,13- <i>O</i> -diacetate (XVII)	388	0.59 (blue)	26.3	29.5
Trinervita-1(15),8(19)-dien-2 β ,3 α ,9 α -triol-2,3- <i>O</i> -diacetate (XVIII)	404	0.42 (greenish blue)	27.1	28.8
13-Oxotrinervita-1(15),8(19)-dien-2 β ,3 α -diol-2,3- <i>O</i> -diacetate (XIX)	402	0.56 (violet)	27.7	29.0
Unknown U1	416	0.59 (orange)	27.8	30.1
Unknown U2	418	0.28 (green)	27.9	30.6
Trinervita-1(15),8(19)-dien-2 β ,3 α ,9 α -triol-2,3,9- <i>O</i> -triacetate (XX)	446	0.52 (orange)	27.9	31.0
Trinervita-11(12),15(17)-dien-3 α ,9 β ,13 α -triol-9- <i>O</i> -acetyl-3,13-dipropionate (XXI)	474	0.63 (purple)	29.8	32.0
Trinervita-11(12),15(17)-dien-3 α ,9 β ,13 α -triol-3,9,13- <i>O</i> -tripropionate (XXII)	488	0.60 (purple)	30.5	33.6

^aTLC on silica gel G, solvent ethyl acetate:dichloromethane (5:95, v/v). Stained by vanillin.

^b1.6 m \times 3 mm 3% OV-101 on 100-120 Gas Chrom Q; Retention indices relative to *n*-alkanes.

^c1.6 m \times 3 mm 3% OV-17 on 100-120 Gas Chrom Q.

TABLE 4. RELATIVE AMOUNTS OF DEFENSIVE SECRETION IN *Nasutitermes*

Species	Monoterpenes (μg)	Diterpenes (μg)	Others (μg)	Diterpene- monoterpene ratio	Percent secretion ^a
<i>Nasutitermes longinasus</i> (minor)	23-37	110-150		4.1:5.4	10-14
<i>Nasutitermes longinasus</i> (major)	46-76	232-360		4.0:5.5	11-17
<i>Nasutitermes havilandi</i>	3.8-6.6	18-47	0.2-0.8	4.7:9.5	2.4-3.8
<i>Nasutitermes johoricus</i>	1.0-1.1	10.1-14.5	0.1-0.6	10.1:13.2	1.4-2.0
<i>Nasutitermes matangensis</i>	4.0-7.0	20.0-33.0	0.6-2.0	4.5:6.2	1.3-1.8
<i>Nasutitermes species 01</i>	22-31	105-138		4.5:4.8	6.4-7.6

^aPercent based on body weight of termite soldier.

molecular formula $\text{C}_{24}\text{H}_{36}\text{O}_4$, shows characteristic fragment ions corresponding to the elimination of one and two molecules of acetic acid. The IR spectrum shows a strong band at 1732 cm^{-1} (ester $\text{C} = \text{O}$). The ^1H NMR (Table 5) exhibited three methyl, two acetate methyl, and three olefinic proton signals. The ^{13}C NMR spectra (Table 6) show two acetyl groups, a quaternary, seven methine, six methylene, and four olefinic carbons. Some characteristic carbon absorptions of the diterpene XVII at $\delta 38.31(\text{C}1)$, $33.81(\text{C}2)$, $80.14(\text{C}3)$, $48.61(\text{C}4)$, $36.27(\text{C}5)$, $30.23(\text{C}6)$, $36.80(\text{C}7)$, $129.69(\text{C}11)$, $131.56(\text{C}12)$, $73.47(\text{C}13)$, $37.44(\text{C}14)$, $148.41(\text{C}15)$, $60.02(\text{C}16)$, $112.49(\text{C}17)$, and $19.83(\text{C}18)$ match quite closely with $38.43(\text{C}1)$, $35.52(\text{C}2)$, $79.44(\text{C}3)$, $48.14(\text{C}4)$, $36.15(\text{C}5)$, $30.01(\text{C}6)$, $36.79(\text{C}7)$, $123.84(\text{C}11)$, $135.89(\text{C}12)$, $73.24(\text{C}13)$, $37.44(\text{C}14)$, $146.89(\text{C}15)$, $59.43(\text{C}16)$, $112.9(\text{C}17)$, and $19.71(\text{C}18)$, respectively, of the known tripropionate XXII from *N. matangensis*. The ^1H NMR data show characteristic exocyclic $\text{C}(17)\text{H}_2$ absorptions and the 16-H doublet, but the 11-olefinic proton appears as a broad flat-top peak (half-width ca. 18 Hz) which could accommodate a doublet-doublet of ca. 6-10 Hz. The broadening of 11-H, not present in compounds XXI and XXII, may be due to the lack of an acetate/propionate group at the C-9 position, allowing for a limited rotation of the C(8)-C(12) bridge in the vicinity of the exocyclic olefin bond. Two protons with α -acetoxy groups have comparable chemical shifts and coupling constants as those of previously described compounds XXI

TABLE 5. COMPARISON OF [¹H]NMR (100 MHz) SPECTRA OF DITERPENES (CDCl₃)^a

Proton	XIV	XV	XVI	XVII	XXI	XXII
H-3		5.26dd (9,8)	5.07dd (8,8)	5.12dd (5,11)	5.14dd (4.4, 11.7)	5.14dd (4.4, 11.7)
H-6	5.63brs	5.65brs	5.60brs			
H-9	5.63brdd (3,3)	5.7brdd (3,3)	5.75brdd (3,3)		4.9m	5.07brdd (6.3, 10.7)
H-11				5.66m ^b	5.29t (8.3)	5.39t (8.3)
H-13				4.91brdd (5, 12)	4.9dd (4.4, 11.7)	4.91dd (4.4, 11.7)
H-14	4.99brddd (3, 3, 3)	4.89brddd (3, 3, 3)	4.90brddd (3, 3, 3)			
H-16	2.31brd (12)	2.72brd (16)	2.45brd (16)	2.53d (12)	2.52brd (11.0)	2.60brd (11.0)
H ₂ -17				4.8brs 4.9brs	5.02brs 5.03brs	5.06brs 5.07brs
Me-17	1.05s	1.01s	0.99s			
Me-18	1.18s	1.11s	1.23s	0.9s	0.93s	0.91s
Me-19	1.77d (1.5)	1.81d (1)	1.81d (1)	0.83d (5.6)	0.86d (6.5)	0.86d (6.4)
Me-20	0.84d (5)	0.84d (6)	0.85d (6)	1.5brs	1.65brs	1.65brs
CH ₃ COO	2.02s	2.04s 2.06s	2.04s 2.08s	2.02s 2.03s	2.03s	
CH ₃ CH ₂ COO					2.3q (7.3)	2.3q (7.3)
CH ₃ CH ₂ COO					1.12t(6H) (7.3)	1.12t(9H) (7.3)

^abr, s, d, t, and m = broad, singlet, doublet, triplet, and multiplet, respectively; coupling constants (Hz) in parenthesis.

^bBoth 100- and 200-MHz spectra show a broad flat-top peak with half-width of 18 Hz; possibly a broadened dd of ca. 6–10 Hz.

and XXII. Based on the above information, the diterpene can tentatively be assigned as trinervita-11(12),15(17)-dien-3 α ,13 α -diol-3,13-*O*-diacetate (XVII). The [¹³C]NMR data for compounds XIV, XV, XVI, and XVII are listed in Table 6 for comparative purposes.

The two major diterpenes, XXI and XXII, isolated from extracts of *N. matangensis*, have been reported previously without the name of the species being established (Prestwich et al., 1981c), and we now present their complete GC, TLC, and NMR data (Tables 3, 5, and 6).

Several studies (Prestwich et al., 1979b, 1981a,c; Braekman et al., 1980,

TABLE 6. COMPARISON OF [¹³C]NMR (25 MHz) SPECTRA OF DITERPENES (CDCL₃)

Carbon (multiplicity)	XIV	XV	XVI	XVII	XXI	XXII
C-1(d)	39.13	39.48	39.83	38.31	38.39	38.43
C-2(t)	36.27	29.42	29.60	33.81	33.48	33.52
C-3(d)	214.64(s)	74.88	72.65	80.14	79.46	79.44
C-4(s)	53.82	45.45	44.92	48.61	48.04	48.14
C-5(t)	44.34	40.65	44.92	36.27	36.17	36.15
C-6(d)	124.78	125.36	124.19	30.23(t)	30.02(t)	30.01(t)
C-7(s)	132.73	133.50	133.44	36.80(d)	36.75(d)	36.79(d)
C-8(s)	144.44	144.38	146.25	52.94(d)	44.24(d)	44.22(d)
C-9(d)	131.86	129.63	130.51	40.60(t)	74.13	74.11
C-10(t)	24.74	25.27	25.32	33.40	29.67	28.31
C-11(d)	55.04	55.51	54.51	129.69	123.86	123.84
C-12(d)	26.08	29.96	28.72	131.56(s)	135.91(s)	135.89(s)
C-13(t)	40.59	40.95	40.89	73.47(d)	73.61(d)	73.24(d)
C-14(d)	71.31	72.13	72.24	37.44(t)	37.40(t)	37.44(t)
C-15(s)	40.36	40.01	40.54	148.41	146.85	146.89
C-16(d)	70.20	68.15	64.81	60.02	59.39	59.43
C-17(q)	17.31	19.18	17.78	112.49(t)	113.97(t)	112.90(t)
C-18(q)	24.91	26.38	27.72	19.83	19.67	19.71
C-19(q)	22.46	22.69	26.38	21.23	11.77	11.90
C-20(q)	19.71	19.83	19.89	19.18	11.59	11.75
CH ₃ COO(s)	170.18	170.94	170.41	170.12	170.90	
		170.70	170.41	170.82		
CH ₃ COO(q)	21.17	21.17	21.35	21.52	21.19	
		21.52	22.69	22.81		
EtCOO(s)					173.53	173.45
					174.35	174.27
						174.27
CH ₂ CH ₃ (t)					28.27	27.78
					27.92	27.78
						27.78
CH ₂ CH ₃ (q)					9.14	9.12
					9.14	9.12

1983, 1984; Dupont et al., 1981; Baker and Walmsley, 1982; Gush et al., 1985; Valterova et al., 1986) have documented that considerable interspecific variation occurs in the monoterpene and diterpene distribution for 15 *Nasutitermes* species from various parts of the world. This documentation and discriminator studies have shown the possible use of such distribution as chemotaxonomic characters (Prestwich, 1983) in establishing the interrelationships within this genus. In this respect, it is noteworthy that the current studies (Table 7) of the chemical defense secretions of *Nasutitermes* as reported here

TABLE 7. INTERSPECIFIC DISTRIBUTION OF DITERPENES FROM *Nasutitermes* SPECIES^a

Termites	Trinervitane			Rippertane, R ₁			Secotrinermitane, S ₂			Kempene		
	T ₁	T ₂	T ₃	R ₁	S ₂	K ₁	K ₂	K ₃				
<i>N. haviladi</i>		+										
<i>N. johoricus</i>	+	++	+++ ^b									
<i>N. longinasus</i> mj. mi.	+	+++	+									
<i>N. matagensis</i>		+	+++									
<i>N. species 01</i>		++	+									
<i>N. columbicus</i>	+	+++										
<i>N. corniger</i>	+	+++										
<i>N. ephratae</i>		++		+								
<i>N. nigriceps</i>	+	+++		+								
<i>N. octopilis</i>	+	++										
<i>N. gagei</i>	+	+++		+								
<i>N. costalis</i>		+++										
<i>N. rippertii</i>	+	+++		+								
<i>N. princeps</i>									++			
<i>N. gracilirostris</i>			+++									
<i>N. sp. N.D.</i>	++									+		
<i>N. kemneri</i>			+++									
<i>N. lujae</i>	+	++							+			
<i>N. infuscaus</i>	+	++	+									
<i>N. kempae</i>		+	+									+++

^aRelative percent: ++++, >40%; ++, 20-40%; +, 5-20%; first five listings—present work. T, R, S, L, and K refer to trinervitane, rippertane, secotrinermitane, longipane, and kempene, respectively; subscripts refer to degree of oxygenation of the diterpene skeleton.

^bTentative.

will eventually contribute towards the better understanding of the taxonomic position of the genus in relation to the other genera in the subfamily Nasutitermitinae. The generic separation of the species within the subfamily, based mainly on morphological characteristics of the soldiers, is still far from being satisfactory. For example, the basis for generic distinction between *Bulbitermes* and *Nasutitermes* is based largely on the constricted head capsule in *Bulbitermes* soldiers. As demonstrated in this study, the major chemical components of the defense secretion of *Nasutitermes havilandi* come close to that of *Bulbitermes singaporensis* (Prestwich et al., 1981b). On the other hand, *Nasutitermes matangensis* would appear to be distinct from these two species. A comparison of the chemistry of the different species of these genera may in many ways provide further insight into the phylogenetic relations of such species and genera.

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ALLELOPATHY IN SALINE AGRICULTURAL LAND: VEGETATION SUCCESSIONAL CHANGES AND PATCH DYNAMICS

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Abstract—In reclamation fields of salt-affected wasteland, five plant communities colonized the undisturbed land, represented by *Cynodon dactylon*, *Desmostachya bipinnata*, *Prosopis juliflora*, *Sporobolus arabicus*, and *Suaeda fruticosa*. Kallar grass (*Leptochloa fusca*), a highly salt tolerant plant when cultivated, shared dominance with *Cynodon*, *Desmostachya*, and *Sporobolus* in 15-month-old fields, whereas *Polypogon* was the only dominant species in 30-month-old kallar grass fields. Through successional stages, soil pH, salinity, sodicity, and Na, K, Ca + Mg significantly decreased due to leaching. Electrical conductivity successively changed from 13.0 to 3.0 to 1.0, while soil total nitrogen, NH₄ nitrogen, NO₃ nitrogen and available P significantly increased. In high-density kallar grass fields, six weed species appeared only in well-defined patches and radially eliminated or reduced kallar grass growth. Many soil factors, such as pH, EC, NH₄ nitrogen, NO₃ nitrogen and available P analyzed in patch vegetation soils, were mostly either comparable or significantly better than those of surrounding kallar grass fields. On the other hand, aqueous extracts of all six invading species and kallar grass significantly reduced kallar grass seed germination to varying degrees. Further, decaying leaf powder of allelopathically suspected species significantly reduced kallar grass biomass, which varied from species to species and in most cases corresponded with field data of kallar grass in patch vegetation. It should be strongly pointed out that allelopathic behavior discussed in patch

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dynamics was in areas where soil saline-sodic conditions had improved greatly (e.g., EC = from 13.0 to only 1.0) due to kallar grass plantation. Further, *Suaeda* appeared to be a poor competitor when soil conditions improved for other species as well, and it could not capitalize on its evolutionary strategic trait of performing well in saline-sodic conditions. To our knowledge, this is the first report indicating that allelopathy may be a factor in determining growth and distribution of plants in saline or sodic soils.

Key Words—Revegetation, reclamation, salinity, succession, kallar grass, *Leptochloa fusca*, nutrients, allelopathy.

INTRODUCTION

Due to agricultural practices, salinity is a rapidly growing global problem, predominantly where agriculture has been prominent for centuries. It is paradoxical that the irrigation canal system in Pakistan has resulted in salinization and/or water-logging of irrigated soils, which now exist as wastelands. Reclamation of such wasteland is essential; one effective way is by selecting and growing salt-tolerant plant species (Chaudri et al., 1964; Kleinkopf et al., 1975; Sandhu and Malik, 1978; Wallace et al., 1982; Naqvi, 1983).

Salt-affected soils are by no means devoid of vegetation. An ecological understanding of such vegetation may provide some useful information regarding the nature and intensity of salinization (Bodhla et al., 1981; Rutter and Sheikh, 1962). Subsequently, new species of economic importance may be introduced. Sandhu and Malik (1975) successfully introduced kallar grass [*Leptochloa fusca* (L.) Kunth.] in these wastelands for increased plant establishment and biomass production. Kallar grass is highly tolerant of salt (Sandhu et al., 1981) and sodicity (Aslam et al., 1979).

Even though the cultivation of kallar grass has clearly improved soil conditions (Malik, 1978), there were no systematic studies found in the literature describing the vegetation and soil properties of the saline lands and successive changes after kallar grass planting. With this in mind, a project was designed to document: (1) vegetation and soil composition of undisturbed areas, (2) successive changes after planting kallar grass and its own productivity, (3) invasion of kallar grass fields by various species, (4) patch dynamics of invading species, and (5) their allelochemic dominance in kallar grass fields. Nomenclature follows Stewart (1972), unless otherwise noted.

METHODS AND MATERIALS

Survey of Vegetation. The studies were carried out at the Biosaline Research Station (BSRS) of the Nuclear Institute for Agriculture and Biology, Faisalabad. BSRS is located near the village of Dera Chahl situated on Bedian Road, 30 km

from Lahore. The study site had not been cultivated for at least 40 years and remained free of human activities, chiefly due to the salinity. There may have been minor grazing by small wild animals. Therefore, this site will be referred as the undisturbed wasteland. For vegetational analysis, study plots were roughly delimited at five different sites within undisturbed wasteland by their dominant species without reference to the subordinate species. Four line transects, each 20 m long, were placed at random at each site and cover of species along each transect was recorded. Each community was named after the species most representative of the cover. For distribution of weed species in kallar grass fields, a quadrat method was used. Twenty quadrats (1-m² size) were randomly placed in fields where kallar grass had been grown for 15 or 30 months. Percent frequency (F) and density (D) (number of plants per quadrat) of weed species were recorded. Importance values (IV) were calculated as $IV = (F + D)/2$.

During our sampling of kallar grass fields, it was surprisingly obvious that the invading weeds nearly always appeared in well-defined dense patches surrounded by kallar grass. To describe this observation quantitatively, five patches of each of such weeds were located and quantified by a line transect method to indicate their range of invasion in kallar grass fields. Cover of weed species and kallar grass was recorded.

To determine kallar grass biomass, four 0.25-m² quadrats were randomly located in 15- and 30-month-old fields of kallar grass, and shoot biomass was harvested and dried to constant weight.

Soil Analysis. Four soil samples were collected from each site at the 0- to 15-cm level for physical and chemical analysis of the soil. Soil texture was determined by the hydrometer method (Bouyoucos, 1962) and water holding capacity by the Keen-Reckzowski box method (Piper, 1942). Soil pH was determined by a glass electrode pH meter, electrical conductivity (EC) by the Solu bridge conductivity meter, Na and K by flame photometry, and Ca + Mg by titration with ethylene diamine tetraacetate (EDTA). CO₃ and HCO₃ were determined by titration with H₂SO₄, and Cl by titration with AgNO₃ (USDA, 1954). Soil samples were extracted with 2 N KCl and analyzed for NH₄ and NO₃ by steam distillation with Devarda's alloy and MgO (Bremner and Keeney, 1965), and total nitrogen was determined following Bremner (1965). Available phosphorus was determined by the Olsen method (Watanabe and Olsen, 1965), and total phosphorus was determined after digestion with perchloric acid (Ogner et al., 1984).

Allelopathy Studies: Effects of Invading Species on Kallar Grass Seed Germination. To determine the effects of six major species invading kallar grass fields, aqueous extracts of each species and kallar grass were prepared and tested against kallar grass seed germination. Plant material was immersed in hot water (approx. 45°C) for 5 min to stop microbial activity before preparing the bioassay extracts. A 10% aqueous extract of each species was prepared by soaking the shoot material in water for 30 min at 25°C, and diluting to 2.5 and 5.0 (w/

v) percent solutions for experimentation. Final pH of all dilutions was adjusted to 6.0. Fifty kallar grass seeds were planted on filter paper in a Petri plate in triplicate and watered with various concentrations of extracts of all invading species. Seeds were allowed to germinate in the dark at a constant temperature of 25°C. Controls were treated in the same manner, except Petri plates were watered with distilled water. Germination was terminated after six days and recorded as percent germination.

Effects of Decaying Leaves of Invading Species on Kallar Grass Growth. Air-dried shoot material of each of six invading species and kallar grass was mixed in soil at a rate of 2 g/100 g soil and placed in 10-cm-diameter plastic pots. Shoot material was allowed to decay for two weeks and planted with four stubbles of kallar grass in each of four pots for each species. Seedlings were allowed to grow for four weeks, harvested, oven dried, and biomass recorded.

RESULTS

Vegetation. Five plant communities dominated by *Desmostachya bipinnata*, *Sporobolus arabicus*, *Suaeda fruticosa*, *Cynodon dactylon*, and *Prosopis juliflora* were recognized in the non-kallar grass fields in this study (Table 1). Apart from the dominant species, one or more of these five species were found

TABLE 1. MEAN PERCENTAGE COVER OF PLANT SPECIES IN DIFFERENT COMMUNITY TYPES

Species	Community type				
	<i>Desmostachya bipinnata</i>	<i>Sporobolus arabicus</i>	<i>Suaeda fruticosa</i>	<i>Prosopis juliflora</i>	<i>Cynodon dactylon</i>
<i>Desmostachya bipinnata</i>	62.04	1.25		1.55	
<i>Sporobolus arabicus</i>	8.54	37.58	10.16	2.66	10.50
<i>Suaeda fruticosa</i>	1.96	6.24	33.74	2.33	10.75
<i>Prosopis juliflora</i>			7.87	61.11	1.00
<i>Cynodon dactylon</i>	0.16	0.67	10.01	19.55	47.05
<i>Kochia indica</i>		1.66	1.50		2.00
<i>Acacia modesta</i>	0.50	1.21	0.50		
<i>Chenopodium album</i>		0.25			
<i>Polypogon monspeliensis</i>		0.16	0.12		
<i>Cyperus rotundus</i>			0.62		0.25
<i>Spergula rubra</i>			0.12		2.37
<i>Dichanthium annulatum</i>					1.37
<i>Senebiera didyma</i>				1.11	0.12
<i>Capparis decidua</i>				0.44	
Bare	26.80	50.98	35.36	11.25	24.59

to be codominant. Kallar grass-introduced fields were invaded by weeds over a 30-month period (Table 2). *C. dactylon*, *D. bipinnata*, *S. arabicus*, and *S. fruticosa* were prominent weeds in 15-month-old kallar grass fields, while *Polypogon monspeliensis* was the principal invader followed by *C. dactylon*, *Kochia indica*, *Cnicus arvensis*, *Cyperus rotundus*, *Scirpus martius*, *Rumex dentatus* and *Spergula rubra* in 30-month-old fields (Table 2).

Six major weeds invaded established kallar grass fields in well-defined patches. Kallar grass growth was very poor and stunted when growing in association with the invading weeds. However, its vigor improved gradually away from the patch boundaries. Kallar grass biomass in 30-month-old fields was significantly lower than in 15-month-old fields (Table 3).

Soils. The soils in undisturbed sites were generally saline-sodic, and the degree of salinization varied for different plant communities (Figure 1, Table 4). The soils under *Suaeda* were highly saline and sodic, whereas those under *Desmostachya* and *Sporobolus* were moderately saline and highly sodic. *Cynodon* dominated slightly saline and moderately sodic soil, and *Prosopis* nonsaline but moderately sodic soils. The soils associated with *Cynodon* were relatively fine textured and had a higher water-holding capacity compared with soils dom-

TABLE 2. CHANGES IN MEAN IMPORTANCE VALUES OF PLANT SPECIES AT DIFFERENT SUCCESSIONAL STAGES

Species	Undisturbed	Kallar grass	
		15 months	30 months
<i>Polypogon monspeliensis</i>	0.04	1.62	21.47
<i>Desmostachya bipinnata</i>	23.42	6.37	
<i>Sporobolus arabicus</i>	16.42	4.93	0.75
<i>Suaeda fruticosa</i>	12.08	4.66	0.75
<i>Cynodon dactylon</i>	8.61	3.95	1.94
<i>Spergula rubra</i>	0.33	0.93	0.75
<i>Cyperus rotundus</i>	0.19	0.46	0.87
<i>Kochia indica</i>	1.04		0.87
<i>Conyza ambigua</i>			1.50
<i>Cnicus arvensis</i>			0.75
<i>Rumex dentatus</i>			0.75
<i>Blumea membranacea</i>		0.23	
<i>Acacia modesta</i>	0.61		
<i>Dichanthium amnulatium</i>	0.17		
<i>Senebiera didyma</i>	0.61		
<i>Chenopodium album</i>	0.03		
<i>Prosopis juliflora</i>	13.99		

TABLE 3. CHANGES IN SOIL PROPERTIES AT DIFFERENT SUCCESSIONAL STAGES ($N = 5$, MEAN \pm SE)

Soil characteristics	No kallar grass (undisturbed)	Kallar grass	
		15 months	30 months
Biomass (g/0.25 m ²)	—	229.0 \pm 24.16	85.3 \pm 4.09 ^c
pH	10.05 \pm 0.11	9.14 \pm 0.24 ^a	8.85 \pm 0.09 ^b
EC, (mS/cm)	13.08 \pm 5.01	2.92 \pm 0.37 ^a	1.12 \pm 0.07 ^{b,c}
Ca + Mg (meq/liter)	3.65 \pm 0.24	3.00 \pm 0.58	1.88 \pm 0.35 ^b
Na (meq/liter)	136.75 \pm 22.4	57.95 \pm 8.52	14.88 \pm 1.14 ^{b,c}
K (meq/liter)	1.26 \pm 0.10	0.59 \pm 0.16 ^a	0.38 \pm 0.03 ^b
Sodium adsorption ratio	100.55 \pm 15.2	47.45 \pm 7.30 ^a	15.34 \pm 1.27 ^b
Exchangeable sodium percentage	59.50 \pm 6.1	40.74 \pm 5.71	17.65 \pm 2.10 ^{b,c}
Total N (ppm)	202.50 \pm 25.62	467.50 \pm 35.75 ^a	400.00 \pm 21.98 ^b
NH ₄ (ppm)	7.47 \pm 0.92	14.35 \pm 3.40	13.13 \pm 1.68 ^b
NO ₃ (ppm)	2.02 \pm 0.45	4.35 \pm 0.51 ^a	1.75 \pm 0.20 ^{b,c}
Total P (ppm)	117.93 \pm 25.84	177.75 \pm 14.77	188.00 \pm 23.10
Olsen P (ppm)	17.01 \pm 2.50	7.78 \pm 0.77 ^a	9.92 \pm 0.85 ^b

^aSignificantly different (t value, ≤ 0.05 or better) between undisturbed and 15-month-old field.

^bSignificantly different (t value, ≤ 0.05 or better) between undisturbed and 30-month-old field.

^cSignificantly different (t value, ≤ 0.05 or better) between undisturbed and 15-month-old fields and 30-month-old fields.

inated by the rest of the species. All soils were alkaline. The predominant anions were CO₃ and HCO₃ (Table 4).

Through successional stages, soil conditions improved remarkably from natural to 15-month- and 30-month-old kallar grass fields. Importantly, soil pH, salinity sodicity, Na, K, and Ca + Mg, significantly decreased, whereas soil total nitrogen, NH₄-N, NO₃-N, and available phosphorus significantly increased in 15-month- and 30-month-old kallar grass fields (Table 3). Electrical conductivity significantly correlated with pH, Na, K and available P, but not with Ca + Mg, NO₃-N, NH₄-N, total N, and total P ($r = 0.91, 0.97, 0.80, 0.71, 0.57, 0.32, -0.51, -0.66, -0.33$, respectively). Consequently, all factors correlated with EC also improved as EC decreased from 13.0 to 2.9 to 1.1 in three respective chronological periods (Table 3).

The soil conditions in patch vegetation associated with kallar grass fields did not show any definite trends to explain reduced kallar grass growth. Soils associated with *Desmostachya*, *Kochia*, and *Sporobolus* were moderately saline and highly sodic, and soils under *Polypogon* and *Suaeda* were nonsaline and nonsodic. *Cynodon* soils were nonsaline and moderately sodic. Soil pH values in all patches were comparable to surrounding kallar grass fields with some significant variations (Table 5). pH values in *Suaeda* and *Polypogon* and EC

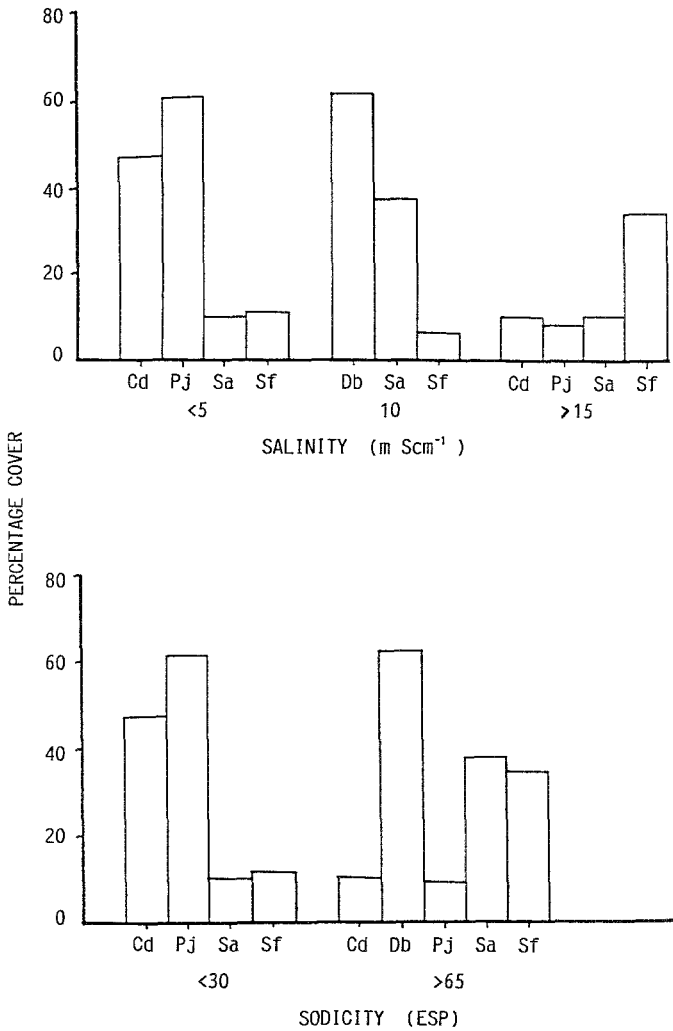


FIG. 1. Percentage cover of different plant species in relation to soil salinity and sodicity. Cd = *Cynodon dactylon*, Db = *Desmostachya bipinnata*, Pj = *Prospis juliflora*, Sa = *Sporobolus arabicus*, Sf = *Suaeda fruticosa*.

values in *Cynodon* and *Polypogon* were significantly lower than in kallar grass fields (Table 5). Electrical conductivity in *Suaeda* patch soils was not significantly different when compared with kallar grass soils. Available phosphorus and available nitrogen ($NH_4 + NO_3$) were always higher in patch soils than in kallar grass soils. Of the two available nitrogen sources measured, NO_3-N was always significantly higher in patch soils, whereas NH_4-N was lower in two

TABLE 4. SOIL ANALYSES OF UNDISTURBED PLANT COMMUNITIES (MEAN \pm SE)

Soil factors	Community type				
	<i>Desmostachya bipinnata</i>	<i>Sporobolus arabicus</i>	<i>Suaeda fruticosa</i>	<i>Propis juliflora</i>	<i>Cynodon dactylon</i>
Sand (%)	61.6 \pm 4.53	57.0 \pm 2.71	61.0 \pm 4.72	67.0 \pm 2.71	55.0 \pm 1.90
Salt (%)	24.3 \pm 2.87	28.0 \pm 1.31	26.0 \pm 2.10	18.6 \pm 0.95	25.5 \pm 1.71
Clay (%)	13.9 \pm 0.69	15.0 \pm 1.20	13.0 \pm 1.12	14.3 \pm 0.71	19.50 \pm 0.85
Textural class	Loam to Sandy Sandy loam	Loam to Sandy Clay loam	Loam to Sandy loam	Loam to Sandy Clay loam	Clay Loam to Sandy Clay loam
Water-holding capacity (% soil oven dry wt)	28.7 \pm 1.32	29.3 \pm 0.95	31.9 \pm 0.95	31.6 \pm 1.73	36.94 \pm 1.00
pH	10.1 \pm 0.14	9.6 \pm 0.13	9.6 \pm 0.20	7.8 \pm 0.03	8.5 \pm 0.45
EC (mS/cm)	10.8 \pm 1.84	10.1 \pm 1.50	15.0 \pm 1.72	2.3 \pm 0.52	4.72 \pm 1.42
Soluble cations (meq/liter)					
Ca + Mg	2.3 \pm 0.13	1.7 \pm 0.37	1.9 \pm 0.39	1.00 \pm 0.04	2.6 \pm 0.81
K	1.5 \pm 0.10	1.0 \pm 0.25	2.6 \pm 0.56	0.90 \pm 0.25	1.0 \pm 0.25
Na	180.0 \pm 12.33	99.0 \pm 11.7	139.8 \pm 27.1	13.2 \pm 2.05	26.25 \pm 3.10
Sodium adsorption ratio	170.2 \pm 10.87	126.9 \pm 9.71	181.8 \pm 20.61	26.6 \pm 3.58	24.71 \pm 3.21
Exchangeable sodium percentage	71.4 \pm 3.92	65.0 \pm 5.73	73.5 \pm 2.53	22.5 \pm 3.05	25.8 \pm 4.12
Soluble aminos (meq/liter)					
Cl	23.47 \pm 5.16	32.9 \pm 1.10	40.8 \pm 3.49	5.4 \pm 0.90	10.5 \pm 2.52
HCO ₃	11.99 \pm 1.26	23.7 \pm 6.41	39.2 \pm 5.44	6.2 \pm 0.96	10.4 \pm 2.20
CO ₃	47.33 \pm 10.47	36.6 \pm 7.94	24.3 \pm 7.66	3.6 \pm 0.27	3.33 \pm 2.10

TABLE 5. ANALYSIS OF SOILS FROM PATCH VEGETATION AND COMPARISON WITH SURROUNDING KALLAR GRASS FIELDS (N = 4, MEAN ± SE)

Sites	pH	EC	Amount (ppm)		
			NH ₄ -N	NO ₃ -N	Available P
Kallar grass	9.14 ± 0.24	2.92 ± 0.37	14.35 ± 3.40	4.35 ± 0.51	7.78 ± 0.77
<i>Suaeda</i>	8.57 ± 0.17 ^a	3.27 ± 0.26	15.71 ± 2.01	6.83 ± 1.47 ^a	14.79 ± 1.47 ^a
<i>Cynodon</i>	9.06 ± 0.11	1.91 ± 0.14 ^a	26.95 ± 2.70 ^a	7.70 ± 2.87 ^a	17.30 ± 3.46 ^a
<i>Desmostachya</i>	9.78 ± 0.07 ^a	7.16 ± 2.38 ^a	8.75 ± 2.24 ^a	7.53 ± 2.78 ^a	19.09 ± 3.17 ^a
<i>Sporobolus</i>	10.41 ± 0.03 ^a	9.46 ± 1.14 ^a	3.68 ± 0.44 ^a	16.10 ± 2.11 ^a	17.35 ± 2.54 ^a
<i>Kochia</i>	10.39 ± 0.03 ^a	12.34 ± 1.04 ^a	8.72 ± 0.81 ^a	9.63 ± 1.75 ^a	22.21 ± 1.89 ^a
<i>Polypogon</i>	8.71 ± 0.8 ^a	1.23 ± 0.02 ^a	4.55 ± 0.61 ^a	5.25 ± 0.83 ^a	11.80 ± 1.15 ^a

^a *t* values significantly different from kallar grass values at $P \leq 0.05$ level.

cases of patch vegetation when compared with kallar grass soils (Table 5). Overall, patch soil chemistry was more hospitable than the surrounding kallar grass fields, whereas the percent cover of kallar grass was severely reduced in all patches, with maximum reduction in the *Cynodon* patch followed by the *Suaeda*, *Desmostachya*, *Kochia*, *Sporobolus*, and *Polypogon* patches (Table 6).

TABLE 6. PERCENTAGE COVER OF PLANT SPECIES OF PATCH VEGETATION IN KALLAR GRASS FIELDS (N = 5, MEAN ± SE)

Species	Patch vegetation					
	<i>Polypogon</i>	<i>Kochia</i>	<i>Suaeda</i>	<i>Cynodon</i>	<i>Desmostachya</i>	<i>Sporobolus</i>
<i>Polypogon monspeliensis</i>	40.0 ± 1.5					
<i>Kochia indica</i>		60.4 ± 6.8				
<i>Suaeda fruticosa</i>			76.5 ± 4.8	0.79 ± 0.5	1.3 ± 1.3	1.0 ± 1.0
<i>Cynodon dactylon</i>	2.00 ± 2.0		3.8 ± 1.0	79.4 ± 4.3	1.2 ± 1.2	1.5 ± 1.5
<i>Desmostachya bipinnata</i>					71.8 ± 5.4	
<i>Sporobolus arabicus</i>			2.9 ± 1.1	3.2 ± 3.2	0.8 ± 0.6	61.8 ± 2.2
<i>Leptachloa fusca</i>	46.41 ± 3.8	20.2 ± 5.4	9.6 ± 2.2	8.8 ± 1.7	14.3 ± 3.0	21.2 ± 1.1
<i>Cyperus rotundus</i>				4.1 ± 2.8		
Bare	11.6 ± 3.9	19.4 ± 6.6	7.2 ± 2.0	3.8 ± 1.5	10.6 ± 3.1	14.4 ± 3.2

Allelopathy Studies. Aqueous extracts of five of the six invading species and kallar grass were significantly inhibitory to kallar grass seed germination, at least at one test concentration (Figure 2). *Cynodon* extracts were an exception, where kallar grass seed germination was not significantly different from control. Kallar grass seed germination was affected significantly in varying degrees depending on the species, extract concentrations, species \times concentration, and overall factors (Figure 2).

Decaying material from shoots of all six invading species, kallar grass, and farm manure significantly reduced shoot dry weight of kallar grass (Table 7). Maximum growth inhibition of kallar grass was caused by *Suaeda*, followed by kallar grass, *Desmostachya*, *Cynodon*, *Sporobolus*, *Kochia*, *Polypogon*, and farm manure (Table 7). Degree of inhibition of kallar grass varied significantly between allelopathically invading species and, in most cases, corresponded with the field data of kallar grass cover in patch vegetation (Tables 6 and 7).

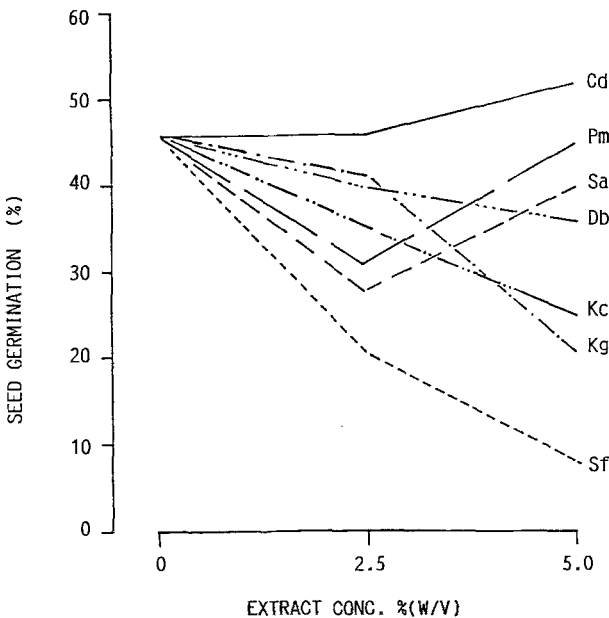


FIG. 2. The effects of aqueous extracts of different species on kallar grass seed germination. ANOVA, $P \leq 0.05$ or better; species LSD = 13.8; concentration LSD = 5.25; species \times concentration LSD = 19.63; overall LSD = 13.88. Legends in addition to those in Figure 1, Pm = *Polypogon monspeliensis*, Kc = *Kochia indica*, Kg = kallar grass.

TABLE 7. EFFECTS OF DECAYING SHOOT MATERIALS OF DIFFERENT PLANT SPECIES ON GROWTH OF KALLAR GRASS ($N = 4$, MEAN = mg/plant)

Treatment	Shoot dry wt. ^a	% of control
Control	257.50e	
Farm manure	193.50d	75.29
<i>Cynodon</i>	121.00b	47.08
Kallar grass	101.25b	39.39
<i>Kochia</i>	137.75bc	53.59
<i>Polypogon</i>	177.25cd	68.96
<i>Suaeda</i>	45.75a	17.80
<i>Desmostachya</i>	117.25b	45.62
<i>Sporobolus</i>	132.25b	51.45

^aMeans followed by the same letter are not significantly different, Duncan's multiple-range test. $P \leq 0.05$.

DISCUSSION

Vegetation of an area is governed by a complex of environmental factors, both physical and biological. In a particular area, climate controls the general distribution of vegetation, whereas microenvironmental conditions determine if a species can survive. Soil plays an important role in this regard as it affects plant growth and distribution.

Cynodon dactylon, *Desmostachya bipinnata*, *Prosopis juliflora*, *Sporobolus arabicus*, and *Suaeda fruticosa* were dominant in the five community types recognized in undisturbed areas (Table 1). *Cynodon* had appreciably high cover in slightly saline and moderately sodic soils (Figure 1, Table 4). The species may extend into high salinity areas, although with smaller cover values (Rutter and Sheikh, 1962; Sheikh and Irshad, 1980). Although slight salinity on the surface does not appear to harm *Cynodon*, salinity in the root zone disturbs its nutritive functions, causing a reduction in yield (Malik et al., 1984).

The soils under *Prosopis* were nonsaline and moderately sodic (Figure 1, Table 4). Other *Prosopis* species are reported to dominate salt- and sodium-free soils (Kayani and Sheikh, 1981). The lower salinity and sodicity may be due to enhanced leaching of salts in the *Prosopis* covered area as the sodium adsorption ratio was significantly lower at the center of tree canopies than in soil between trees (Virginia and Jarrell, 1983). *Desmostachya* was restricted to moderately saline but highly sodic soils and had a high cover value (Figure 1, Table 4). The species is known to occur on soils with a range of salinities from 2.7 to 27.0 mS/cm (Rutter and Sheikh, 1962). *Desmostachya* is an arid zone species, normally occupying nonsaline soils, but it also manages to tolerate or

avoid salinity. The species has a wide ecological amplitude and cannot be depended upon as an indicator of specific soil conditions (Malik et al., 1984).

Sporobolus arabicus was a common species throughout the study area (Table 1). The soils of the *Sporobolus* community were moderately saline and highly sodic (Figure 1, Table 4). The species is reported to occupy a wide range of soils with reference to salinity and sodicity (Rutter and Sheikh, 1962; Sheikh and Mahmood, 1986). Occasionally, *Sporobolus* may grow where salinity exceeds 35 mS/cm, although with smaller cover values than those it attains at low salinity (Rutter and Sheikh, 1962; Malik et al., 1984). Saline and nonsodic soil conditions seem more hospitable for *Sporobolus* (Sheikh and Mahmood, 1986).

Suaeda has been reported to be dominant on very highly saline (45–80 mS/cm) and sodic soils (Rutter and Sheikh, 1962, Malik et al., 1984). The species constitutes the main natural vegetation on saline-sodic sand dunes and is also present on soils free from salinity and sodicity (Din and Farooq, 1975). The species was also found at low salinity, although with smaller cover values than those it attained at high salinity, suggesting that *Suaeda* is a poor competitor when soil conditions were improved for other species (Figure 1, Table 4). Sheikh and Mahmood (1986) reported that soil under *Suaeda* was marginally saline because of leaching of salts to a lower depth in the soil profile. Additionally, low soil surface salinity may be due to the fact that a dense stand of *S. fruticosa* can accumulate approximately 2.5 tons/hectare of NaCl annually (Chaudhri et al., 1964). Clearly, salinity does seem to play a major role in distribution of plant species in the area and correlates with several soil factors (Table 4).

In general, the undisturbed soils were saline, sodic, and alkaline (Table 4). A direct relationship between pH and sodicity of soil has been suggested (Fireman and Wadleigh, 1951; Rutter and Sheikh, 1962). The presence of high concentrations of CO_3 and HCO_3 seem responsible for high alkalinity. If soils are affected by sodium and potassium salts capable of alkaline hydrolysis, e.g., NaHCO_3 and Na_2CO_3 , sodicity and alkalinity will occur together (Szabolcs, 1979).

Soil pH, salinity, and sodicity appreciably decreased, while total nitrogen, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, and available phosphorus increased in 15- and 30-month-old kallar grass fields (Table 3). Such nutritional changes through succession have been reported in natural communities (Rice et al., 1960) and in surface coal mining areas (Lodhi, 1979), allowing nonannual species to invade and revegetate these areas. Similarly, kallar grass fields were successively invaded by plant species not recorded in undisturbed fields, in addition to the species found in undisturbed areas (Table 2). Chaudhri (1952) reported that the vegetation of the fields under reclamation was a mixture of exotic species and natural flora, the latter being replaced by the former depending on the degree of reclamation.

The species invading kallar grass fields clearly appeared in dominant

patches and radially eliminated or reduced kallar grass cover in all patches, with lowest cover in the *Cynodon* patch followed by *Suaeda*, *Desmostachya*, *Kochia*, *Sporobolus*, and *Polypogon* patches (Table 6). On the other hand, soils associated with various vegetational patches did not show any definite trend with nutritional status. In *Cynodon* and *Polypogon* patches, where kallar grass cover was the lowest and the highest respectively, the associated soil pH and EC were significantly lower than the surrounding kallar grass fields (Table 5). However, EC and pH in *Desmostachya*, *Sporobolus*, and *Kochia* patches were significantly higher than in kallar grass fields. Further, salinity–sodicity factors were not unsuitable for kallar grass growth because the species is highly tolerant to salinity (Sandhu et al., 1981) and sodicity (Aslam et al., 1979). Additionally, available P, $\text{NH}_4\text{-N}$, and $\text{NO}_3\text{-N}$ amounts were always significantly higher in all patches as compared to kallar grass field soils, with the exception of $\text{NH}_4\text{-N}$ in *Sporobolus* and *Polypogon* patches which were significantly lower (Table 5). Thus, the elimination of kallar grass from the weed patches cannot be attributed to the soil factors analyzed, while it failed to persist in patch-associated species (Table 5, 6).

Plant interactions are known to play an important role in distribution and coexistence of species in both natural and agroecosystems. Competition for necessary growth factors (allelopathy) and addition of toxic substances to the environment (allelopathy) can play a major role in plant growth (Szczepanski, 1977; Rice, 1984). Our preliminary data indicated, based on soil analyses, that competition may not be the leading cause of kallar grass elimination from patch vegetation. On the other hand, aqueous extracts and decaying leaf material of all six invading species and kallar grass significantly reduced kallar grass seed germination (Figure 2) and shoot growth (Table 7). The allelopathic influence on kallar grass varied from species to species and in most cases clearly corresponded with field data (Table 6, 7). It should be strongly pointed out that allelopathic behavior discussed in patch dynamics was in areas where soil saline–sodic conditions had improved greatly (review Table 3, and correlations discussed in results) due to kallar grass cultivation. Further, *Suaeda* appeared to be a poor competitor (Table 2) when soil conditions improved for other species as well, and it could not capitalize on its evolutionary strategy of performing well in high saline–sodic conditions. Therefore, growth inhibition in kallar grass caused by *Suaeda* was due to allelopathy, perhaps somewhat accentuated by ions in its shoot tissue. Our ongoing research indicates that relatively ion-free *Suaeda* extract retained its allelochemic influence. In general, species competing for a limited growth factor can allelopathically influence the growth of other species to minimize competition (Tremmel and Peterson, 1983; Rice, 1984). Such interactions can only accentuate the invasion by incoming species. Detailed investigations are in progress to ascertain the relative importance of patch dynamics relating to kallar grass susceptibility to competition and allelopathy.

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EVIDENCE FOR (*E,Z*)-8,10-DODECADIENYL
ACETATE AS THE MAJOR COMPONENT
OF THE SEX PHEROMONE OF THE
EASTERN PINE SEEDWORM,
Cydia toreuta (Lepidoptera: Tortricidae)

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Abstract—The sex pheromone of *Cydia toreuta* (Grote), the eastern pine seedworm, was investigated using electroantennogram (EAG) measurements, gas chromatography (combined GC-FID-EAD) measurements, and field tests. (*E,Z*)-8,10-dodecadienyl acetate (*E,Z*8,10-12:Ac) and (*E,E*)-8,10-dodecadienyl acetate (*E,E*8,10-12:Ac) produced both the highest EAG and EAD responses. Only a single antennal stimulatory peak was observed when female abdominal tip extracts were analyzed by GC-FID-EAD, which corresponded exactly with that of authentic *E,Z*8,10-12:Ac. Field tests confirmed *E,Z*8,10-12:Ac as the major pheromone component. The *E,Z* isomer by itself caught equivalent numbers of male moths as did caged females. The addition of the *E,E* isomer to the *E,Z* form increased trap catch, although not significantly ($P = 0.05$). The addition of (*Z,Z*)-8,10-dodecadienyl acetate to the *E,Z* isomer significantly ($P = 0.05$) reduced trap catch, while the addition of (*Z,E*)-8,10-dodecadienyl acetate to the *E,Z* form also decreased trap catch, but not significantly ($P = 0.05$).

Key Words—Sex pheromone, *Cydia toreuta*, Lepidoptera, Tortricidae, Olethreutinae, (*E,Z*)-8,10-dodecadienyl acetate.

INTRODUCTION

The eastern pine seedworm, *Cydia* (= *Laspeyresia*) *toreuta* (Grote) feeds on the seeds of several North American conifers including jack pine, *Pinus banksiana* Lamb.; loblolly pine, *P. taeda* L.; lodgepole pine *P. contorta* Dougl.; red pine, *P. resinosa* Ait.; shortleaf pine, *P. echinata* Mill.; and Virginia pine, *P. virginiana* Mill. (Hedlin et al., 1981). Usually, one to three larvae inhabit a cone, but there may be 12 or more in cones of some host trees (Lyons, 1957). Each larva generally consumes 4–15 seeds (Ciesla and Bell, 1966; Mattson, 1978). Last-instar larvae overwinter within the cone axis, with pupation taking place the following spring. Male emergence slightly precedes that of the females, and emergence is normally completed within a two-week period during May and/or June. Little is known about adult behavior.

All identified sex attractants in the Tortricidae have been mono- or diunsaturated straight-chain alcohols, acetates, or aldehydes. A review of identified sex attractants revealed that, with few exceptions, the subfamily Olethreutinae responds to compounds with a 12-carbon chain length (Roelofs and Brown, 1982). Furthermore, all species investigated within the genus *Cydia* are attracted to compounds within the 12-carbon chain series (Roelofs et al., 1971; Dix et al., 1984; Greenway, 1984; Chisholm et al., 1985; Sartwell et al., 1985; Stevens et al., 1985). Chisholm et al. (1985) conducted a field screening test in a forested area northeast of Saskatoon, Canada, in which all four geometrical isomers of 7,9- and 8,10-dodecadienes with acetate, alcohol, and aldehyde functional groups were utilized. *C. toreuta* males were caught in sticky traps baited with (*E,Z*)-8,10-dodecadienyl acetate (*E,Z*8,10-12:Ac). Using those results as a starting point, this paper further investigates the sex pheromone of *C. toreuta* using a solvent extract of the pheromone glands of virgin female moths, electroantennogram (EAG) investigations with synthetic 10-, 12-, and 14-carbon compounds, and field tests with possible attractants.

METHODS AND MATERIALS

Insects and Pheromone Collection. *C. toreuta* adults were obtained from field-collected mature red pine cones that contained diapausing last-instar larvae. Cones were collected during January and February 1987 and were maintained under a 16:8 light-dark photoperiod at 24°C. Emergence began ca. 25 days after exposure to laboratory conditions. Pheromone was collected from 1- to 3-day-old virgin females 1–2 hr after the beginning of scotophase. The ovipositor was forcibly everted and, along with the terminal three abdominal segments, excised and extracted with dichloromethane for 30 min. Extracts from

28 females were collected ca. one week before use, divided into two equal portions and each was reduced under a stream of N_2 to $<2 \mu\text{l}$. Further, two groups of 10 females each were excised the same day that they were to be used for gas chromatographic analysis and the extracts likewise reduced under a N_2 stream.

Analyses. A gas chromatograph (GC) (Hewlett Packard 5710) equipped with both a flame ionization detector (FID) and an electroantennogram detector (EAD) (combined GC-FID-EAD) (Arn et al., 1975) was used to detect pheromone components eluting from the column and analyzing synthetic standards. The effluent split between detectors was ca. 70:30, favoring the FID. The GC was equipped with a DB-5 column (bonded methyl 5% phenyl silicone 30 m \times 0.32 mm), and injected samples were ramped from 40 to 90°C in 30 sec, and then programmed from 90 to 230°C at 2°C/min. Helium carrier gas linear velocity was 22.9 cm/sec. (at 150°C).

Electroantennogram (EAG) and Synthetic Chemicals. Syntheses and chromatographic purification of chemicals used for the EAG studies and the field trials were carried out in the laboratory (Plant Biotechnology Institute, Saskatoon, Canada) as previously described (Chisholm et al., 1985). All had a purity of 97–99+%. Isomers (*E,E*; *E,Z*; *Z,E*; *Z,Z*-8,10-dodecadienyl acetates) that had high EAG activity were further assayed for purity using the GC-FID-EAD. This was done by GC analysis of 5 ng of each isomer. Pure compounds were recognized by a single appropriate FID peak and a corresponding single EAD peak, or no EAD peak for compounds that were not stimulatory.

The antennal response of *C. toreuta* to synthetic chemical standards was measured by EAG as previously described (Chisholm et al., 1975). Synthetic standards of 12-carbon monounsaturated and all isomeric 8,10-diunsaturated acetates, alcohols, and aldehydes were tested at least three times and 10- and 14-carbon monounsaturated acetate compounds twice. Values for air blanks applied to antennae were subtracted from values for test standards. All standards were tested using 1 μg applied to filter paper disks.

A dose-response was run on antennae using *E,Z*8,10-12:Ac. Dosages ranged from 1×10^{-6} to 10 μg applied to filter paper disks.

Field Studies. Pherocon 1C traps were used for field trapping and contained rubber septa (A.H. Thomas No. 8753-D22) that had been impregnated with synthetic chemical lures. Two drops of a 10% solution of the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BHT) in acetone were added to each septum. In addition, one treatment consisted of two virgin females placed in separate cages attached to the inside roof of a trap. Females were replaced daily with freshly emerged individuals. Previous field trapping with caged females indicated that maximum calling occurred within 24 hr after emergence. Tests were conducted in the field from May 5 to May 30, 1987, during the flight period of *C. toreuta*.

Traps were placed in a 17-year-old red pine stand near the city of New Richmond, St. Croix County, Wisconsin. Trees were ca. 6 m in height. Traps were hung at a height of 3–4 m on the end of an exposed branch at the edge of the crown. Individual traps were placed ca. 20 m apart. Field experiments were set out in a randomized complete-block design, with three blocks, each block separated by ca. 100 m. Moth captures were recorded daily and trap bottoms were replaced as required. Traps within blocks were rerandomized every second day. Catch/trap treatment data were transformed ($\sqrt{x + 1}$), analyzed by an analysis of variance (ANOVA), and significantly different means were separated by Duncan's (1955) multiple-range test ($P = 0.05$).

RESULTS

EAG Profile. The greatest antennal responses to monounsaturated 10-, 12-, and 14-carbon acetates were elicited by *E*10–12:Ac (3.35 ± 0.6 mV above background, $N = 6$), *Z*10–12:Ac (2.8 ± 0.6 mV, $N = 7$), and *E*8–10:Ac (2.4 ± 0.4 mV, $N = 2$). Other relatively high responses were elicited by *E*8–12:Ac (1.75 ± 0.3 mV, $N = 10$), *E*9–12:Ac (1.7 ± 0.4 mV, $N = 4$), *Z*8–12:Ac (1.6 ± 0.2 mV, $N = 9$), and *Z*9–12:Ac (1.6 ± 0.5 mV, $N = 4$). All other monounsaturated compounds, including the 12-carbon alcohols and aldehydes, gave lesser (< 1.0 mV) responses.

The greatest overall antennal responses for this study were elicited by the 8,10 diunsaturated 12-carbon acetates, namely, *E,Z*8,10–12:Ac (7.6 ± 0.5 mV above background, $N = 15$), *E,E*8,10–12:Ac (7.55 ± 0.8 mV, $N = 8$), *Z,E*8,10–12:Ac (4.9 ± 0.7 mV, $N = 5$) and *Z,Z*8,10–12:Ac (4.6 ± 0.9 mV, $N = 5$). Other strong responses were recorded for *E,Z*8,10–12:Ald (3.2 ± 0.3 mV, $N = 5$) and *E,E*8,10–12:Ald (2.3 ± 0.3 mV, $N = 5$). All other diunsaturated compounds gave lesser (< 2.0 mV) responses.

Antennal recovery times were not measured precisely; however, it was clearly noticeable that recovery time of an antenna following exposure to *E,Z*8,10–12:Ac was much greater than for any other tested compound.

The response of male antennae to varying concentrations of the *E,Z* isomer was investigated using the EAG (Figure 1). At dosages as low as 1×10^{-4} μ g antennal responses were > 1 mV.

GC-FID-EAD. Injection of a mixture of synthetic 8,10–12:Ac isomers, 5 ng each, resulted in strong antennal stimulation in response to both the *E,E* and *E,Z* isomers and a lesser response to the *Z,E* isomer (Figure 2). There was no discernible EAD response elicited by the *Z,Z* isomer. When the four isomers were injected individually (Figure 3), each gave a single FID peak, indicating high isomeric purity of the synthetic material. However, single EAD responses

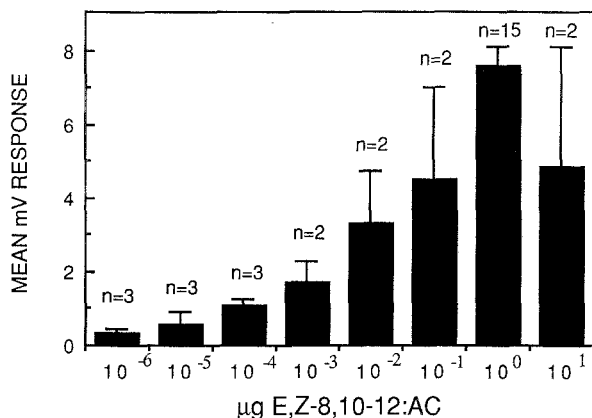


FIG. 1. Mean mV responses from EAG recordings of *C. toreyuta* male antenna to *E,Z,8,10-12:Ac* dosages ranging from 1×10^{-6} to $10 \mu\text{g}$. Error bars indicate standard error.

were observed that corresponded with the injected parent compound only for the *E,Z* and *E,E* isomers. When the *Z,E* isomer was injected, EAD responses corresponding to both the *Z,E* and *E,Z* forms were observed. In the case of the *Z,Z* isomer, no EAD response was elicited that corresponded with the parent compound; rather, a response corresponding with the retention time of the *E,Z* isomer was observed.

Only a single pheromone component was detected when female abdominal tip extracts were analyzed by GC-FID-EAD (Figure 2). In each of four analyses, a single antennal stimulatory peak was observed that had a retention time that corresponded exactly with that of authentic *E,Z,8,10-12:Ac*. No other EAD peaks were consistently present in the analyses.

Field Tests. Table 1 shows the 1987 field tests of traps baited with 8,10-12-carbon acetates. Significant differences between means were indicated when ANOVA was applied to transformed data ($MSE = 12.9$, $F = 20.1$, 11 *df* for treatment and 22 *df* for error, $P < 0.001$). The highest catches per trap were recorded for the mixture of *E,Z,8,10-12:Ac* ($200 \mu\text{g}$) + *E,E,8,10-12:Ac* ($20 \mu\text{g}$) with a mean of 613.0 moths/trap. However, there were no statistical differences in mean catch per trap between that lure composition and the *E,Z,8,10-12:Ac* ($200 \mu\text{g}$) + *E,Z,8,10-12:Ac* ($2 \mu\text{g}$), *E,E,8,10-12:Ac* ($200 \mu\text{g}$), *E,Z,8,10-12:Ac* ($50 \mu\text{g}$), and/or caged females. The *E,E,8,10-12:Ac*, *Z,E,8,10-12:Ac*, and the *Z,Z,8,10-12:Ac* isomers alone did not catch moths in a quantity greater than the blank traps. The *Z,Z* isomer combined with the *E,Z* isomer significantly ($P = 0.05$) reduced trap catch when compared to *E,Z*

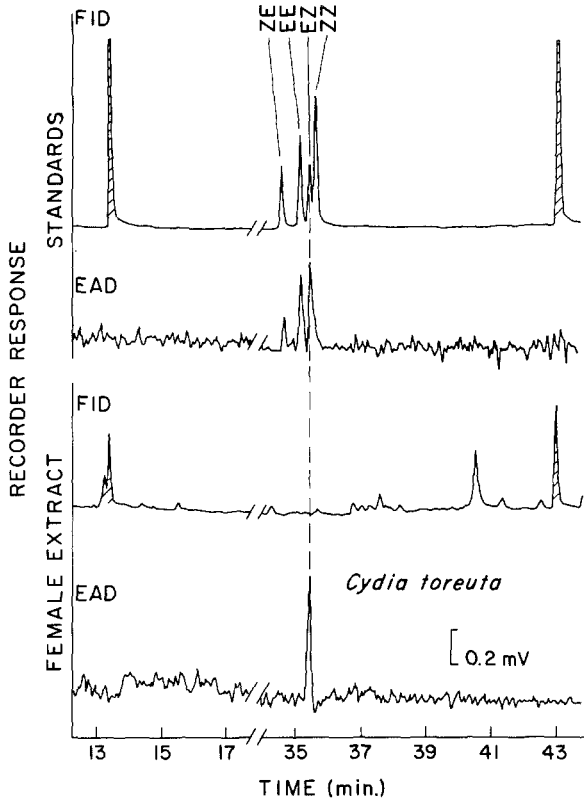


FIG. 2. Paired GC-FID-EAD chromatograms recorded from male *C. toreuta* antenna subjected to (top) a standard isomer mixture of *Z,E*-, *E,E*-, *E,Z*-, and *Z,Z*-dodecadienyl acetates, 5 ng of each; (bottom) a female abdominal tip extract (14 FE). Cross-hatched peaks are internal standards decyl alcohol and tetradecyl acetate, respectively. Conditions: DB-5 column 30 m \times 0.32 mm, programmed from 90 to 230°C/min, splitter ratio was 70:30 favoring FID.

+ *E,E* isomer combinations as well as to the *E,Z* form alone. The addition of *Z,E* isomer to the *E,Z* isomer significantly ($P = 0.05$) reduced trap catch when compared to *E,Z* + *E,E* mixtures, but not compared to the *E,Z* form alone.

DISCUSSION

Based on the EAG study both *E,E*8,10-12:Ac and *E,Z*8,10-12:Ac appeared to be good candidates for major sex pheromone components. Both compounds elicited strong EAG responses of about equal value, although the *E,Z* isomer

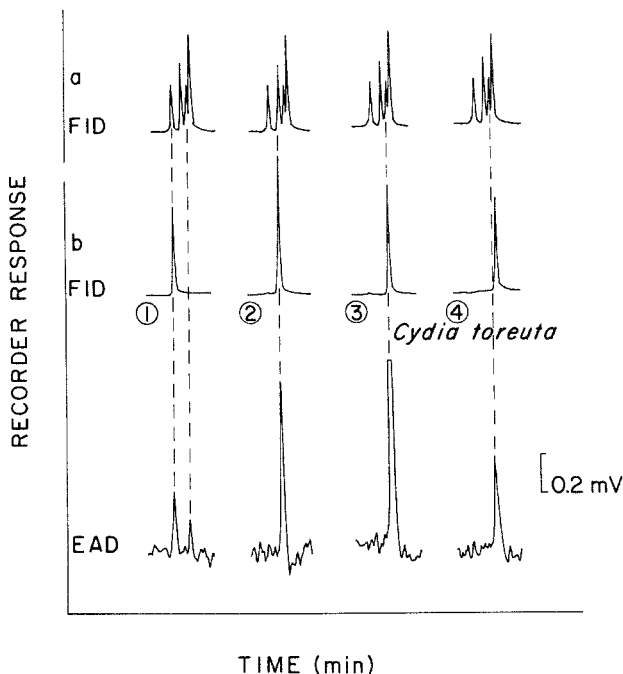


FIG. 3. FID and EAD responses to synthetic 8,10-dodecadienyl acetates, 5 ng of each. (a) Flame ionization detector (FID) responses to mixed isomers; order of elution is *Z,E*; *E,E*; *E,Z*; and *Z,Z*. (b) FID and electroantennogram (EAD) responses to individual injections of (1) *Z,E*; (2) *E,E*; (3) *E,Z*; and (4) *Z,Z* isomers. A trace impurity of the *E,Z* isomer in both the *Z,E* (1) and the *Z,Z* (4) compounds was detected by the EAD response. Except for the *Z,Z* isomer (4), each compound elicited an EAD response. Conditions: DB-5 column 30 m × 0.32 mm, programmed from 90 to 230°C at 4°C/min, splitter ratio was 70:30 favoring FID.

EAG response trace exhibited a much slower return to baseline. In many lepidopterous species the EAG produced by the attractant has a much slower recovery time than the other test chemicals; however, in other studies inhibitors have been characterized by a much slower return to baseline (Roelofs and Comeau, 1971).

The purity of the geometrical isomers of 8,10-12:Ac was investigated with the GC-FID-EAD after all gave strong EAG responses of >4.5 mV. This was done because conjugated dienes are not stable and are known to isomerize (Shani and Klug, 1980; Ideses et al., 1982; Chisholm et al., 1985). For the *Z,E* isomer, two EAD peaks were detected. One peak corresponded to the *Z,E* isomer FID peak, while the other eluted at the proper time for the *E,Z* isomer,

TABLE 1. CAPTURES OF ADULT MALE *C. toreuta* IN TRAPS BAITED WITH DIUNSATURATED 12-CARBON COMPOUNDS AND CAGED FEMALES, MAY 5-30, 1987

Lure composition (μg)	Catch/trap \pm SE and significance ^a
<i>E,Z</i> ,10-12: Ac (200)	428.0 \pm 135.3ab
<i>E,Z</i> ,10-12: Ac (50)	411.0 \pm 42.3ab
<i>E,E</i> ,10-12: Ac (200)	7.0 \pm 4.0d
<i>E,E</i> ,10-12: Ac (50)	2.3 \pm 0.3d
<i>Z,E</i> ,10-12: Ac (200)	8.0 \pm 1.5d
<i>Z,Z</i> ,10-12: Ac (200)	3.3 \pm 0.9d
<i>E,Z</i> ,10-12: Ac (200) + <i>E,E</i> ,10-12: Ac (2)	571.3 \pm 209.1a
<i>E,Z</i> ,10-12: Ac (200) + <i>E,E</i> ,10-12: Ac (20)	613.0 \pm 173.3a
<i>E,Z</i> ,10-12: Ac (200) + <i>Z,E</i> ,10-12: Ac (20)	197.3 \pm 62.9bc
<i>E,Z</i> ,10-12: Ac (200) + <i>Z,Z</i> ,10-12: Ac (20)	113.3 \pm 16.4c
Caged females	373.3 \pm 23.2ab
Blank trap	1.0 \pm 0.6d

^a Means within a column followed by the same letter are not significantly different ($P = 0.05$).

thus indicating that a small contamination of the *E,Z* form was present although it was too small for the FID to detect. For the *Z,Z* isomer only a single EAD peak was detected; however, that peak corresponded to the *E,Z* isomer elution time. Again, the contamination was too small for the FID to detect. In the case of the *Z,Z* isomer the presence of a small quantity of the *E,Z* form may have influenced the EAG results by increasing the mV response noted for the *Z,Z* isomer, especially since the *Z,Z* isomer itself did not appear to elicit an EAD response. However, a strong response from the *E,Z* isomer contaminant on the EAD may have influenced the antennal response to the *Z,Z* isomer, which eluted 0.10 min after the *E,Z* isomer. In addition, *E,Z* isomer contamination within the *Z,E* isomer may have increased the EAG response obtained for the *Z,E* form. The dose-response curve indicated that *E,Z* dosages as low as 1×10^{-4} μg could elicit EAG responses > 1 mV. Both the *E,E* and *E,Z* isomers appeared to be highly pure. However, since the *Z,Z* isomer did not elicit an EAD peak, its presence within the *E,E* or *E,Z* material could not be precluded.

Further, EAD analysis of female extracts indicated that the *E,Z* isomer is a pheromone component within the female abdominal tip. However, the amount of *E,Z* in 14 female equivalents was undetectable on the FID. Although the male responds strongly to *E,E*, this compound was not detected in the female abdominal tip.

Field tests confirmed *E,Z*,10-12: Ac as the major component of the sex pheromone of *C. toreuta*. The *E,Z* isomer by itself caught statistically equiva-

lent numbers of moths as female baited traps. The *E,E* isomer, which by itself is unattractive, may act as a synergist, since addition of *E,E* to *E,Z* increased trap catch, although not significantly. The *Z,E* and *Z,Z* isomers, when combined with the *E,Z* form, both caused reduced trap catch and therefore appear inhibitory. Roelofs and Comeau (1971) speculated that inhibitors would be compounds similar to the attractant, which both the *Z,E* and *Z,Z* isomers are.

The evidence presented strongly implicates *E,Z*,8,10-12:Ac as the major component of the sex pheromone of *C. toreuta*. *E,Z*,8,10-12:Ac by itself or in combination with small quantities of *E,E*,8,10-12:Ac provides a pheromone bait that is as attractive as virgin females and can be used for field sampling. Further investigation is needed to correlate economic insect damage/infestation with adult male captures using synthetic pheromone baited traps.

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COMPARATIVE STUDY OF ALLELOPATHY AS EXHIBITED BY *Prosopis juliflora* SWARTZ AND *Prosopis cineraria* (L) DRUCE

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Abstract—The allelopathic effects of *Prosopis juliflora* were studied both in the laboratory and in nature and compared with that of *Prosopis cineraria* to understand the chemical nature of allelochemicals. Both species occupy the same habitats but *P. cineraria* does not appear to have any toxic effect on other plants under its canopy. *P. juliflora* is highly allelopathic and does not allow the growth of any other species. Leaf extracts and leaf leachates of *P. juliflora* were inhibitory. Decaying leaves were also inhibitory at early stages of decomposition. Live roots were not found to be inhibitory in cogermination and interplanting of seeds. Chemical investigation of the extracts showed the allelopathic compounds to be phenolic in nature in both the species. Slow decomposition and heavy accumulation of leaf litter below *P. juliflora* may possibly result in accumulation of toxic substances in soil layers, inhibiting growth of other species.

Key Words—Allelopathy, *Prosopis juliflora*, *Prosopis cineraria*, *Cassia occidentalis*, radish.

INTRODUCTION

Prosopis juliflora Swartz and *Prosopis cineraria* (Linn.) Druce are two common trees of arid and semiarid zones in India. *P. cineraria* is a native slow-growing tree and is beneficial for the growth and development of other species. *P. juliflora*, on the other hand, is an exotic species and grows luxuriantly on sandy soils and is being introduced on a large scale in arid zones because of its

faster growth and soil-binding capacity. It has been realized for some time that *P. juliflora* does not allow the establishment of ground vegetation (Lahiri and Gaur, 1969; Sankhla et al., 1965). Earlier studies on *P. juliflora* suggest that the leaves and the soil below this tree contain inhibitory chemical substances (U. Goel, and G.S. Nathawat, unpublished observations). Allelopathic patterning is clearly visible in the area with heavy accumulation of leaf litter in the case of *P. juliflora*, but *P. cineraria*, despite heavy litter fall, supports much ground vegetation. In earlier studies on the comparative nature of the two species, *P. cineraria* was not found to be allelopathic in laboratory bioassays. In view of this, a comparative study of *P. juliflora* and *P. cineraria* was undertaken to understand the allelopathic effects of the former and to characterize the active principles.

METHODS AND MATERIALS

Species Selection. Field observations revealed that the understory vegetation in the study area is mainly dominated by *Brachiaria ramosa*, *Dactyloctenium indicum*, *Crotolaria medicagena*, *Corchorum tridens*, *Indigofera linnaei*, and *Cassia occidentalis*. The growth of the herbaceous species was observed to be better under *P. cineraria* than under *P. juliflora*. Saxena (1978) recorded the above-ground biomass to be 234.8 g/m² below *P. cineraria*, and bare areas were observed under *P. juliflora*.

Effect of Pericarp, Bark, and Leaves. To determine the most toxic part of *P. juliflora* and *P. cineraria*, pericarp, bark, and leaves were collected from trees in the field and aqueous extracts of 1% and 10% concentrations were obtained by soaking the pericarp, bark, and leaves in hot distilled water for 1 hr, which was considered to be sufficient for extraction. The extracts were suction-filtered through Whatman No. 1 filter paper. Control was maintained by using distilled water.

Twenty-five seeds of radish were planted for germination on moistened filter papers in Petri dishes. The treatments were replicated five times. Germination in this experiment and all subsequent experiments was recorded daily, and radicle and plumule lengths were measured after 10 days. Seedlings were dried for 24 hr at 80°C.

Effect of Field Soil. To determine the stability of toxic compounds in the soil, soil was sampled to a depth of only 10 cm below the canopy of the tree, as most biological activity is encountered close to the surface. The soil was air dried and sieved. Five and ten grams of soil collected from under each tree species was used to make a thin layer under moistened filter papers. Twenty-five seeds of radish and *Indigofera linnaei* were planted for germination in each of the Petri dishes (replicated five times).

Effect of Decomposition of Litter. Field study indicated that *Prosopis* species add 10 g dried leaves per kilogram of 0–10 cm soil in the study area. To test the toxicity of decomposing litter, 15 g of leaf litter was mixed in 2 kg of loam soil and allowed to decay for 8, 4, 2, and 0 weeks prior to the sowing of seeds. Twenty-five seeds of *Cassia occidentalis* were planted in five pots per treatment containing the soil–leaf mixture and watered with tap water when necessary. Seed germination was determined after two weeks and then all pots were thinned to three seedlings per pot. Seedlings were harvested after 16 weeks, and oven dried shoot and root weight was recorded. After the harvest, the soil was tested for residual toxicity by germinating twenty-five seeds of radish on moistened filter papers which were placed on 10 g of soil–litter mixture.

Effect of Age. Fresh leaves of *P. juliflora* and *P. cineraria* from 1-, 6-, and 12-month-old trees were collected from potted plants and 1 g leaf material of each type were spread under the filter paper in Petri dishes. Twenty-five seeds of *C. occidentalis* were planted for germination. Five replicates were maintained for each treatment.

Cogermination of Seeds. To determine the allelopathic effect of roots, 10 seeds of *P. juliflora* were cogerminated with 10 seeds each of *C. occidentalis* and radish. For controls, 20 seeds of each species were germinated.

Growth of C. occidentalis and P. juliflora in Pure and Mixed Stands. To test the toxicity of exudates of live roots, two plants of each species of *P. juliflora* and *C. occidentalis* were planted in pure and mixed cultures in plastic pots of 2-kg capacity containing loam soil. After two months, the plants were harvested, and oven dried weights of roots and shoots were determined.

Extraction and Fractionation of Prosopis Species. One kilogram of air-dried, powdered plant material was extracted in ethyl alcohol for 36 hr. The plant residue was removed by filtering the extract. Alcohol was evaporated and the residue was dissolved in ethyl acetate. The soluble portion was fractionated into acidic, nonacidic, and alkaline fractions by washing it repeatedly with saturated sodium bicarbonate and sodium hydroxide solutions. The bicarbonate soluble portion was acidified to pH 2 with dilute HCl and extracted with ethyl acetate. Similarly, the sodium hydroxide-soluble portion was neutralized with dilute HCl and then extracted with ethyl acetate. All three fractions, bicarbonate, phenolic, and neutral, were washed thoroughly with water and concentrated on a water bath and stored for studying the biological activity. The neutral portion was further fractionated with column chromatography using various solvents as given in Table 8 below. All fractions were concentrated and examined for their biological activity.

Biological Activity. The biological activity of various fractions was determined by dissolving 1 drop of each fraction in 2 ml of water. These solutions were added to Petri dishes containing 25 seeds of radish on filter paper. The germination was recorded according to the procedure described previously.

Aqueous Extract after Extraction with Ethanol. After extracting 10 g of the dried leaf material with ethanol, the residue was filtered, dried, and reextracted with water. The filtrate obtained was made up to 100 ml using distilled water.

The data was statistically analysed using Student's *t* test.

RESULTS

Aqueous Extracts. Seed germination and radicle and plumule growth of radish were significantly inhibited by leaf extracts and 10% concentration of the pericarp and bark extract of *P. juliflora*. In *P. cineraria*, leaf extract and 10% pericarp extract was found to be toxic (Table 1).

The soil was not inhibitory to germination and early seedling growth of the test species (Table 2).

Litter Decomposition. The litter of *P. juliflora* in early stages of decomposition inhibited germination of *Cassia occidentalis* seeds, whereas the 8-week-old decaying litter did not inhibit growth or germination. The dry weight yields of test species were significantly higher in all cases except when the litter was not allowed to decay previously.

The litter of *P. cineraria* did not affect seed germination at any stage, and also the dry weights were higher in all cases as compared to the control (Table 3).

TABLE 1. EFFECT OF LEAF, BARK, AND PERICARP EXTRACTS OF *P. juliflora* AND *P. cineraria* ON GERMINATION AND SEEDLING GROWTH OF RADISH SEEDS^a

	Control	Leaf extract		Bark extract		Pericarp extract	
		1%	10%	1%	10%	1%	10%
<i>P. juliflora</i>							
Germination (%)	100	90		100	100	100	20 ^b
Radicle length (cm)	5.50	0.40		5.10	4.50 ^b	7.60	
Plumule length (cm)	6.82	1.10		6.70	6.22	5.10 ^b	
Dry weight of 10 seedlings (mg)	120	39 ^b		115	94 ^b	110	
<i>P. cineraria</i>							
Germination (%)	100	100		100	100	100	100
Radicle length (cm)	5.50	3.12 ^b		5.40	5.20	5.8	4.87 ^b
Plumule length (cm)	6.82	4.74 ^b		6.78	6.58	6.82	6.28
Dry weight of 10 seedlings (mg)	120	92 ^b		116	110	122	108 ^b

^a Average of 50 seedlings.

^b Significantly different from control at 0.05 level.

TABLE 2. EFFECT OF FIELD SOIL FROM BELOW CANOPY OF *P. juliflora* AND *P. cineraria* ON GERMINATION AND SEEDLING GROWTH OF TEST SPECIES

Concentration (dry material, % g)	Radish			Indigofera linnaei		
	Germination (%)	Radicle length (cm)	Plumule length (cm)	Germination (%)	Radicle length (cm)	Plumule length (cm)
<i>P. juliflora</i>						
Control	100	5.50	6.82	80	1.32	2.50
5	100	7.10 ^b	6.68	80	1.32	2.52
10	100	6.95 ^b	6.98	80	1.33	2.52
<i>P. cineraria</i>						
Control	100	5.50	6.82	80	1.34	2.56
5	100	6.85 ^b	6.98	80	1.32	2.52
10	100	7.10 ^b	6.87	80	1.35	2.56

^a Average of 25 seedlings.

^b Significantly different from control at 0.05 level.

The soil litter mixture did not retain any toxins at the time of harvest (Table 4).

Age Effect and Leaf Decaying. The inhibitory effect of *P. juliflora* during leaf decay was observed on percentage germination followed by its effect on radicle and plumule growth. This effect was not related to age of *P. juliflora*.

In *P. cineraria* inhibition of decaying leaves was not observed; the age of the plants had no effect on germination of the test species (Table 5).

TABLE 3. SEED GERMINATION AND DRY WEIGHT YIELD PER POT OF *C. occidentalis* AS AFFECTED BY DECOMPOSING LITTER OF *P. juliflora* AND *P. cineraria*^a

Decomposition period (weeks)	<i>P. juliflora</i>			<i>P. cineraria</i>		
	Seed germination (%)	Dry weight yield per pot (g)		Seed germination (%)	Dry weight yield per pot (g)	
		Shoot	Root		Shoot	Root
Control	71a	0.490a	0.333a	71	0.490a	0.333a
0	15b	0.459a	0.271b	62	0.987b	0.442b
2	19b	1.016b	0.487c	68	1.118c	0.457b
4	25b	1.224b	0.465c	75	1.225c	0.463b
8	76c	1.162b	0.507c	75	1.212c	0.487b

^a Average of five replicates with three plants per pot. Means not followed by the same letter are significantly different at 0.05 level.

TABLE 4. EFFECT OF SOIL (5 g) MIXED WITH DECOMPOSING LITTER, AFTER 16 WEEKS, ON SEED GERMINATION AND SEEDLING GROWTH OF RADISH

	Control	Soil from pots containing <i>P. juliflora</i> leaves	Soil from pots containing <i>P. cineraria</i> leaves
Germination (%)	100	100	100
Radicle length (cm)	5.6	6.0	6.1
Plumule length (cm)	6.96	7.2	7.15

^a Average of 50 seedlings. Mean values not significantly different from control.

Cogermination and Interplantation. Cogermination of seeds of *P. juliflora* and *C. occidentalis* and radish seeds in Petri dishes did not show any adverse effects of *P. juliflora* on test species. Interplanting *P. juliflora* with *C. occidentalis* in pots gave no indication of the inhibitory nature of the former (Tables 6 and 7).

Fractionation of Ethanolic Extracts of Prosopis Species and Biological Activity. Of all the elutions isolated from the neutral fraction by polar and non-polar solvents, the methanol fraction and phenolics in *P. juliflora* were inhibitory to seed germination and radicle and plumule growth of the test species. Acids were not inhibitory. In *P. cineraria* under laboratory conditions, phenolics, methanol fraction, and acids reduced early seedling growth but the extent of inhibition was less as compared to *P. juliflora* (Table 8).

Water Extract after Ethanolic Extraction. The water fraction derived after

TABLE 5. EFFECT OF DECAYING LEAVES (1 g) FROM DIFFERENT AGED *P. juliflora* AND *P. cineraria* PLANTS ON GERMINATION AND SEEDLING GROWTH OF *C. occidentalis* SEEDS^a

Age	<i>P. juliflora</i>			<i>P. cineraria</i>		
	G (%)	RL (cm)	PL (cm)	G (%)	RL (cm)	PL (cm)
Control	96	2.5	5.8	96	2.5	5.8
1 month	96	1.66 ^b	2.55 ^b	98	2.62	5.67
6 months	96	1.67 ^b	3.15 ^b	98	2.54	5.38
12 months	100	1.66 ^b	2.71 ^b	100	2.48	5.45

^a Average of 50 seedlings. G, germination; RL, radicle length; PL, Plumule length.

^b Significantly different from control at 0.05 level.

TABLE 6. ROOT AND SHOOT YIELD (g) OF *P. juliflora* AND *C. occidentalis* GROWTH FOR TWO MONTHS SEPARATELY AND IN MIXTURE^a

	<i>P. juliflora</i>	<i>P. juliflora</i> + <i>C. occidentalis</i> (2 + 2)	<i>C. occidentalis</i> (4)
Shoot	0.886a	0.581b + 2.147bc	1.344ab
Root	0.354a	0.183b + 0.690bc	0.417ab

^aAverage of five replicates. Means not followed by same letters are significantly different from control at 0.05 level.

TABLE 7. COGERMINATION OF SEEDS OF *P. juliflora* WITH OTHER TEST SPECIES

Species	Germination (%)		Seedling length (cm)	
	<i>P. juliflora</i>	Test species	<i>P. juliflora</i>	Test species
<i>P. juliflora</i> alone	100		7.11 ^a	
Radish alone		100		11.71
<i>C. occidentalis</i> alone		100		7.50
<i>P. juliflora</i> + radish	100	100	7.25	11.05
<i>P. juliflora</i> + <i>C. occidentalis</i>	100	100	6.12 ^b	7.54

^aControl.

^bSignificantly different from control at 0.05 level.

TABLE 8. EFFECT OF VARIOUS SOLVENT FRACTIONS OF *P. juliflora* AND *P. cineraria* ON BIOLOGICAL ACTIVITY OF RADISH SEEDS^a

Solvents used in column for elution	<i>P. juliflora</i>			<i>P. cineraria</i>		
	Germination (%)	Radicle length (cm)	Plumule length (cm)	Germination (%)	Radicle length (cm)	Plumule length (cm)
Control	100	3.36	2.81	100	3.86	2.81
Hexane	100	3.60	2.31	100	3.71	3.45
Benzene	100	2.91	3.60	100	3.00	3.80
Chloroform	100	4.09	4.31	100	3.87	4.08
Methanol	60 ^b	0.92 ^b	1.00 ^b	90 ^b	1.98 ^b	2.25 ^b
Acids	100	2.34	3.85	90 ^b	1.48 ^b	2.20 ^b
Phenolics	70 ^b	1.30 ^b	0.91 ^b	90 ^b	1.88 ^b	1.78 ^b

^aAverage of 25 seedlings.

^bSignificantly different from control at 0.05 level.

TABLE 9. EFFECT OF WATER EXTRACT OF *Prosopis* SPECIES ON RADISH AFTER EXTRACTION WITH ETHYL ALCOHOL^a

	Germination (%)	Radicle length (cm)	Plumule length (cm)
Control	100	2.71	3.22
<i>P. juliflora</i>	100	1.37 ^b	2.39 ^b
<i>P. cineraria</i>	100	1.08 ^b	2.31 ^b

^a Average of 25 seedlings.

^b Significantly different from control at 0.05 level.

extraction of plant material of both the species with ethyl alcohol was most effective in retarding the radicle and plumule growth of the seeds kept for germination (Table 9).

DISCUSSION

It has been hypothesized that leaf litter and canopy leachates greatly influence soil properties like fertility and underground cover (Zinke, 1962; Aggarwal et al., 1976). Chou and Yang (1982) reported that the regulation of understory species in stands of *Phyllostachys edulis* and *Leucaena leucocephala* (Chou and Kuo, 1986) was primarily due to the allelopathic effect of leaf litter. Similarly, in the present studies, leaves seem to influence the understory growth. Leaf extracts in *P. juliflora* were more toxic as compared to *P. cineraria*. In an earlier investigation (U. Goel and G.S. Nathawat, unpublished observations), litter was shown to be highly toxic to seed germination and seedling growth of the test species.

Leaf extracts of *P. cineraria* were significantly toxic in laboratory bioassays. Phenolics, phenolic acids, and the methanolic fraction of the neutral portion were also inhibitory, but the phytotoxins never accumulate to toxic levels in soil. According to Stowe (1979), the experimental conditions of bioassays never adequately simulate natural conditions. Jameson (1970) noted that although phytotoxic effects of *Juniperus osteosperma* leaves could be demonstrated in the laboratory, many associated species were relatively unaffected in the field.

Litter decomposition studies of *P. juliflora* clearly reveal that the inhibitors released to the soil environment are degraded within two months, subsequently leaving no traces of toxicity. In nature, however, litter build-up is a continuous process, and some of it is always in a stage of early decomposition, which is

inhibitory to germination of other species. The litter of *P. cineraria* was non-toxic for germination and seedling growth during decomposition.

Age of the plant is considered to be an important factor as it has been shown that the quantity of inhibitors varies at different ages of *Celosia argentea* (Pandya, 1977), but in *P. juliflora* age of the plant did not alter the inhibitory activity.

Soil texture also plays an important role in allelopathy. In an earlier experiment (U. Goel and G.S. Nathawat, unpublished observations), sandy soil below *P. juliflora* was found to be toxic, and the seedlings of *Indigofera linnaei* had died after two to three days, whereas in the present experiment, the clay soil below the tree was nontoxic to any of the test species. Probably, the retention of moisture in clay tends to have a diluting effect, whereas the sand tends to be dry. The other explanation could be that there is better growth of microorganisms in clay, which may decompose the allelochemicals much faster than in sandy soils. Patrick (1971) also observed greater inhibition of corn height and weight in sandy and light textured soils. Rice (1979) mentioned that phytotoxins can be effective in fine-textured soils also, where they are apparently absorbed and accumulate to toxic proportions.

Oksana et al. (1986) reported water-soluble allelochemicals in *Sorghum bicolor* when cogerminating with the test seeds in Petri dishes and interplanted in pots. No such inhibitory effects were observed in *P. juliflora*, indicating that the live roots were not involved in the production of allelochemicals. The observation that toxicity is evident even after extraction with ethanol, as also shown for many tropical grasses (Chou, 1977), suggests that perhaps more than one toxin is involved in allelopathy.

The studies clearly reveal that phenolic acids and the methanolic fraction of the neutral portion (which may also contain phenolic acids) in combination with nonpolar compounds present in the hexane and benzene fraction may be responsible for inhibiting the germination of seeds and early seedling growth as observed by other workers (Alsaadawi and Rice, 1982; Lodhi, 1978, 1979). It is quite likely that phenolic acids affect higher plant growth through their influence upon soil microorganisms in addition to direct effects on the plants (Rice, 1964). The enhanced allelochemic activity in *P. juliflora* may be ascribed to the combination of major and minor phenolic acids and nonpolar organic compounds that act in synergism with each other. According to Goer and Barney (1968), litter decomposes more rapidly below *P. cineraria* because of better growth of microorganisms. Slow decomposition and heavier accumulation of litter in *P. juliflora* may result in accumulation of toxic substances in soil layers, thus inhibiting growth of other species.

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EUROPEAN CORN BORER SEX PHEROMONE: Structure-Activity Relationships¹

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Abstract—The biological activity of analogs of the pheromone components of the European corn borer, *Ostrinia nubilalis*, (*Z*)- and (*E*)-11-tetradecen-1-ol acetate, in which modifications were made in the terminal alkyl portion were studied in the three pheromonal types of the insect. European corn borer males respond to pheromonal stimuli at three levels of behavioral activity, i.e., short-range sexual stimulation, activation in the flight tunnel, and response in the field. Structural requirements for elicitation of response at these levels were found to be increasingly restrictive, respectively. Flight-tunnel activity was induced only by compounds that had a total chain length of 13 or 14 carbons and in which branching at carbon 13 was limited to one methyl group or a cyclopropyl group. Three new analogs were active in the flight tunnel, viz., (*E*)- and (*Z*)-13-methyl-11-tetradecen-1-ol acetate and (*Z*)-12-cyclopropyl-11-dodecen-1-ol acetate. The cyclopropyl analog was the most active analog against the *ZZ* type of the European corn borer. The *E* isomer, however, was pheromonally inactive in the *EE* type and was shown to be a pheromone antagonist. This dissimilarity is most likely due to differences in structure of the receptors in the European corn borer strains. Analogs that were biologically active against the European corn borer were tested against the redbanded leafroller, *Argyrotaenia velutinana*, which also uses (*Z*)- and (*E*)-11-tetradecen-1-ol acetate as part of its pheromone. Results showed that the redbanded leafroller pheromone acceptor system is different from that of the European corn borer; marginal behavioral response was elicited by only one of the new analogs. Thus, although both species use 11-tetradecen-1-ol acetate isomers as their pheromone, the mechanisms by which they are perceived are different.

¹Mention of a commercial product does not constitute endorsement of that product by USDA.

Key Words—*Ostrinia nubilalis*, Lepidoptera, Pyralidae, redbanded leafroller, *Argyrotaenia velutinana*, Tortricidae, pheromone analogs, sex stimulation bioassay, flight-tunnel behavior, field bioassay, molecular mechanics.

INTRODUCTION

During the past two decades, tremendous strides have been made in the elucidation of the chemistry of insect sex pheromones (Inscoc, 1982; Arn et al., 1986). To make use of this newly gained knowledge in efficient ways there is a need to gain insight into the mechanisms involved in pheromone perception. We conducted this study to provide clues to the spatial and/or functional group requirements for biological activity and to develop a description of pheromone acceptor sites.

The male European corn borer (ECB), *Ostrinia nubilalis*, chemoreceptor system offers an excellent opportunity for such a study. Its female sex pheromone is a simple mixture consisting of the geometrical isomers of 11-tetradecen-1-ol acetate. Furthermore, the ECB has three pheromonal types that use different proportions of the isomers (Klun and Maini, 1979). Also, other moths, such as the redbanded leafroller (RBLR), *Argyrotaenia velutinana*, employ the same compounds as part of their sex pheromone (Bjostad et al., 1985). Thus, the opportunity exists for both intra- and interspecific comparative studies of structure-activity relationships. The compounds involved in this study are shown in Figure 1.

Early on, it was discovered that for the ECB and the RBLR mixtures of (*Z*)- and (*E*)-11-tridecen-1-ol acetate (**3** and **4**) could mimic the pheromone (**1** and **2**) (Klun and Robinson, 1972; Cardé and Roelofs, 1977). Similarly, **3** elicited male response in two tortricid species, *Adoxophyes orana* and *Clepsis spectrana*, which use (*Z*)-11-tetradecen-1-ol acetate as a component of their pheromone (Voerman et al., 1975). Chapman et al. (1978a,b) synthesized the racemate and the optically pure enantiomers of 9-(2-cyclopenten-1-yl)-nonan-1-ol acetate (**5**), a cyclic analog of **1**, and tested male sex stimulation activity of the compounds in the ECB and RLBR. They found that the receptor for (*Z*)-11-tetradecen-1-ol acetate was chiral in the two species but that the manner in which the last four carbon atoms in the pheromone coil within the receptor differs. Bestmann et al. (1980) studied the electroantennogram (EAG) activity of chiral 13-ethyl analogs of (*Z*)-11-tetradecen-1-ol acetate, viz., [*R*-(*Z*)]- and [*S*-(*Z*)]-13-methyl-11-pentadecen-1-ol acetate, the racemate (**6**), and Chapman's cyclopentenyl compounds in the two species of insects. They found that the receptors distinguished between the enantiomers and that the magnitude of EAG activity for the open-chain analogs was greater than that of the cyclopen-






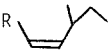

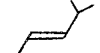



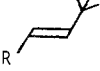

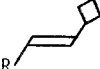
<u>Compound No.</u>	<u>Structure</u>	<u>Chemical name</u>
1		(Z)-11-tetradecen-1-ol acetate
2		(E)-11-tetradecen-1-ol acetate
3		(Z)-11-tridecen-1-ol acetate
4		(E)-11-tridecen-1-ol acetate
5		9-(2-cyclopenten-1-yl)nonan-1-ol acetate
6		(Z)-13-methyl-11-pentadecen-1-ol acetate
7		(Z)-13-methyl-11-tetradecen-1-ol acetate
8		(E)-13-methyl-11-tetradecen-1-ol acetate
9		(Z)-12-cyclopropyl-11-dodecen-1-ol acetate
10		(E)-12-cyclopropyl-11-dodecen-1-ol acetate
11		(Z)-13,13-dimethyl-11-tetradecen-1-ol acetate
12		(E)-13,13-dimethyl-11-tetradecen-1-ol acetate
13		(Z)-12-cyclobutyl-11-dodecen-1-ol acetate
14		(E)-12-cyclobutyl-11-dodecen-1-ol acetate

FIG. 1. Structures and chemical names of compounds assayed for pheromonal activity;
 $R = (CH_2)_{10}OAc$, $R' = (CH_2)_9OAc$.

tenyl analogs. This difference in activity was ascribed to the greater flexibility of the open-chain compounds and to the better opportunity of the acceptor to adapt to the less rigid compound. Because the cyclopentenyl compounds had previously proven to be active in the sex stimulation bioassay, we felt it would be interesting to determine whether the open-chain analogs had greater behavioral activity than the cyclopentenyl analogs. We report the results of these tests and the discovery of new behaviorally active analogs, in which structural modifications were made at the 13 carbon. The bioassay tests were carried out with the three ECB types at three behavioral assay levels, i.e., a short-range sex stimulation assay, upwind response in a flight tunnel, and field trapping. The biological activity of the new analogs was also evaluated against the RBLR.

METHODS AND MATERIALS

Insects. ECB males used in the laboratory bioassays were from colonies that were homozygous for *ZZ* or *EE* female sex pheromone production genes or hybrids obtained by crossing the *ZZ* and *EE* genotypes. The homozygous colonies had been selected from progeny lines of females that were collected in the vicinity of Beltsville, Maryland (Klun and Huettel, 1988). The *ZZ* colony utilized 97:3 (*Z:E*)-11-tetradecen-1-ol acetate as its pheromone, *EE* used 3:97 (*Z:E*), and the *ZE* hybrid used 35:65 (*Z:E*) as its pheromone. Correspondingly, the geometrical proportions of pheromonal analogs tested against males from the respective colonies were always similar to the proportions produced by sibling females. RBLR males were from a colony that was established in our laboratory from eggs supplied to us by W.L. Roelofs, Geneva, New York. All insects were reared on an artificial diet described by Reed et al. (1972). Inasmuch as the ECB and the RBLR are, respectively, nocturnal and crepuscular with regards to their mating activities, the colonies were maintained in environmental chambers under a reversed photoperiod (80% relative humidity; 16 hr light–8 hr dark, 26°C–20°C). This arrangement allowed us to conduct the insect bioassays during normal working hours of the day.

Flight-Tunnel Bioassays. The flight tunnel used in all bioassays was the same as described by Raina et al. (1986). Conditions in the tunnel were: 18–20°C, 40–65% relative humidity, 2.5 lux (red light), and a wind speed (laminar flow) of 50 cm/sec. Most test compounds were applied to a 0.5 × 4-cm filter paper strip in nanogram amounts in 3–5 μ l of heptane, and the strip was positioned on a clip that was suspended on a vertical wire 16 cm from the tunnel floor and 20 cm from the upwind screen-covered end of the tunnel. Males were positioned in a release tube that was 300 cm downwind of the stimulus. When the lid of the release tube was removed, the stimulus plume passed directly through the tube. In one experiment, ECB males were preexposed to phero-

monal analogs and subsequently exposed to a mixture of pheromone and the analog. The preexposure was accomplished by placing a male in the release tube, screened on its upwind end, into the air stream containing the analog evaporated from a paper strip on the clip. The male was preexposed to the analog for 3 min, and then a paper strip treated with pheromone plus analog was positioned on the clip, the screen covering the release tube was removed, and responses to the mixture were observed. In some cases, tests were conducted using rubber septa that were treated with microgram amounts of compound and attached to the clip at the upwind end of the tunnel. Adult males, 2–3 days old, were used in the bioassays after they had been held in the flight tunnel room for ca. 30 min at 2.5 lux and 20°C. Males were tested individually against a treatment and then discarded. Tests with the ECB were conducted during hours 5–6 of scotophase and tests with the RBLR were conducted 1 hr before scotophase or during the first hour of scotophase. In RBLR assays, *E* and *Z* analogs were substituted for the 11-tetradecen-1-ol acetates in the RBLR pheromone mixture identified by Bjostad et al. (1985). All assays were conducted using a randomized complete-block design with replication over time. Each male tested was allowed 3 min to respond after the lid of the release tube was removed. The percentage of males that took flight, oriented upwind in the stimulus plume, and traversed the length of the tunnel in the plume to within 2 cm of the stimulus or landed on it provided a measure of the relative biological potency of each treatment. Treatments referred to as pheromone or natural pheromone throughout this paper are those mixtures of synthetic compounds that are considered to comprise a species' female sex pheromone in nature. The percentage response variables were transformed to arcsin and analyzed by the analysis of variance. Significance was tested by the *F* test, and the means that were significantly different ($\alpha < 0.05$) were separated using the least-significant-difference procedure.

Male Sex Stimulation Assays. Some pheromone analogs did not elicit upwind responses from males in the flight tunnel. The relative biological potency of these compounds was evaluated using a sex stimulation assay. In this assay, a set of five 2- to 3-day-old males in a 6 × 8-cm cylindrical screen cage were positioned in a 50 cm/sec airflow at 19–20°C that was vented outdoors. The set of males was then exposed to 100 ng compound on a 0.5 × 4-cm filter paper that was gently waved 5–6 cm upwind of all males in the cage for 1 min. The number of males that responded to the treatment in the 1-min exposure period with wing fanning, extension of genitalia, and copulatory strikes were recorded for each treatment. A set of five males was exposed to one treatment and discarded. Assays were conducted using a randomized complete-block design with replication over time. The response data were subjected to analysis of variance. Significance was tested using the *F* test, and the significantly different ($\alpha < 0.05$) means were separated using the least-significant-difference procedure.

Field-Trapping Tests. ECB trapping experiments were conducted in the field using a modified *Heliothis* Scentry trap (Scentry, Chandler, Arizona 85224) that has been described (Webster et al., 1986; Klun and Huettel, 1988). In the first test, 10 of the cone-shaped traps were positioned ca. 1 m from the ground and 50 m apart at the perimeter of a cornfield in the vicinity of Beltsville, Maryland. Traps were each randomly baited with a rubber septum that had been treated with 100 μg of compound in 10 μl of heptane. Each compound in the test had previously been demonstrated to cause upwind flight responses in the flight tunnel. The septum was positioned at the center of the circular opening at the bottom of the trap attached to a paper clip and a string that was strung across its diameter. Freshly treated septa were placed in the traps every third night of the test, and male captures were recorded daily over 12 consecutive days.

The second field-trap test involved five treatments that were positioned in the traps using a randomized complete-block design with three replicates. The test was conducted over four consecutive nights and was designed to determine if the addition of a proportionately small amount of the natural pheromone component, (*E*)-11-tetradecen-1-ol acetate (**2**), added to analog **9** would enhance male captures over responses to **9** alone. The test was also designed to determine comparative effectiveness of the geometric mixture with that of a 97:3 (*Z*:*E*) mixture of 11-tetradecen-1-ol acetates. Determination of the rate of evaporation of analog **9** from rubber septa showed (Heath et al., 1986) that it was 0.4 times as volatile as 11-tetradecen-1-ol acetates; therefore, the analog was applied to the septa at a load 2.5 times greater than that of the (*Z*)-11-tetradecen-1-ol acetate.

Chemicals. Compounds **1–4** were commercial products and were purified by preparative high-performance liquid chromatography (HPLC) on two 25-cm \times 10-mm (ID) stainless-steel tubes packed with 10 μm silica impregnated with 20% AgNO_3 using toluene as eluant. Racemic **5** and its enantiomers were supplied by O. Chapman; racemic **6** and its enantiomers were supplied by H.J. Bestmann and O. Vostrowsky. The synthesis of compounds **7**, **8**, **11**, and **12** has been described (Schwarz et al., 1986). Compounds **9** and **10** were prepared as mixtures via the Wittig reaction of (cyclopropylmethylidene)triphenylphosphorane and 11-acetoxyundecanal and were purified by preparative HPLC. Similarly, compounds **13** and **14** were prepared as mixtures from (cyclobutylmethylidene)triphenylphosphorane and 11-acetoxyundecanal, and purified by AgNO_3 HPLC. All new compounds had IR, NMR, and mass spectra fully consistent with the proposed structures.

Minimum Energy Calculations. Minimum energy calculations were performed for the *E* and *Z* isomers of 3-hexene, 2-methyl-3-hexene, 2,2-dimethyl-3-hexene, and 1-cyclopropyl-2-pentene. These compounds represented those parts of the molecule that were altered. The acetate-bearing moiety of the molecules remained constant for all analogs, and was not expected to affect the

total conformation of each molecule differently. Default values for bond angles and bond distances were taken from Streitwieser and Heathcock (1976). The computer program used was the January 1986 version of the CHEMX program written by Chemical Designs Limited, Inc. Energy minimization operations were run for a minimum of 100 cycles in 15-cycle intervals until the change in energy for each compound was less than 0.9 kcal/mol for two consecutive 15-cycle runs.

RESULTS

Response of ZZ-Type Male ECB to Its Female Sex Pheromone and Selected Analogs in Flight-Tunnel and Sex-Stimulation Assays. Results (Table 1) of the sex-stimulation assay of the enantiomers and racemates of **5** and **6** revealed that (\pm)-**5** and (-)-(S)-**5** were twice as active as (\pm)-**6** and (-)-(R)-**6**. Bestmann et al. (1980) have measured the relative EAG activities of these compounds and observed that in their assay (-)-(R)-**6** was 33 times as active as (-)-(S)-**5**. Racemic **5** and **6** and their enantiomers were 0.2–0.4 times as active as the natural pheromone blend. Mixtures of **3** and **4** (50:50 and 97:3), the closely related analog **7**, and a mixture of **7** and **8** (97:3) were ca. 0.7 times as active as the pheromone. In the flight tunnel, no upwind flight response was elicited by **5** or **6**, but the other analogs were active.

Response of Three Male ECB Pheromonal Types to 11-Tetradecen-1-ol Acetates and Its Analogs in Flight Tunnel. When ZZ ECB males were exposed to treatments with their natural pheromonal blend of **1** and **2** (97:3) and the pure Z isomers of **3**, **7**, **9**, and **11**, the upwind flight responses to compounds **3**, **7**, **9** were 59%, 56%, and 53%, respectively, while response to compound **11** was only 5% (Table 2). When EE ECB males were exposed to their natural pheromone blend of **1** and **2** (3:97) and the pure E isomers **8**, **4**, **12**, and **10**, the upwind flight responses to **8**, **4**, **12**, and **9** was 56%, 31%, 12%, and 2%, respectively. When ZE males were exposed to compound **9**, the (Z)-cyclopropyl analog, or to a mixture of **9** and **2**, they showed nearly the same response to the analogs as to the natural hybrid pheromone. The same response was also observed when the ZE males were exposed to compound **1** alone or to a blend of (E)-cyclopropyl compound **10** and **1**. Compound **10** by itself had very low activity, while compound **2** by itself was completely inactive. Similarly, compound **7** alone or in combination with compound **8** in a 40:60 ratio showed activity that was equal to that of the natural hybrid isomer ratio, while the activity of **8** by itself was much lower.

Comparison of Responses of EE-Type Males to Mixtures of 11-Tetradecen-1-ol Acetates and Analogs With and Without Preexposure to Same Analogs in Flight Tunnel. Exposure (Table 3) of EE males to a mixture of the natural pheromones (3:97, Z:E) and **10** resulted in a significant reduction in upwind

TABLE 1. RESPONSE OF ZZ-TYPE MALE EUROPEAN CORN BORER TO ITS FEMALE SEX PHEROMONE AND ANALOGS IN FLIGHT-TUNNEL, SEX-STIMULATION, AND ELECTROANTENNOGRAM (EAG) ASSAYS

Compound	Treatment ^c	Flight tunnel (% upwind flight to stimulus)	Sex stimulation (% response) ^b	Relative EAG ^c
1 + 2	11-tetradecen-1-ol acetate (97:3, Z:E)	100a	85b	100.0
3 + 4	11-tridecen-1-ol acetate (50:50, Z:E)	95a	96a	
3 + 4	11-tridecen-1-ol acetate (97:3, Z:E)	70ab	91ab	
7	(Z)-13-methyl-11-tetradecen-1-ol acetate	53bc	62c	
7 + 8	13-methyl-11-tetradecen-1-ol acetate (97:3, Z:E)	27c	51c	
(S)-6	(Z)-(+)-(S)-13-methyl-11-pentadecen-1-ol acetate	0d	2f	0.10
6	(Z)-(±)-13-methyl-11-pentadecen-1-ol acetate	0d	17e	1.00
(R)-6	(Z)-(-)-(R)-13-methyl-11-pentadecen-1-ol acetate	0d	17c	10.00
(R)-5	(+)-(R)-9-(2-cyclopenten-1-yl)nonan-1-ol acetate	0d	12ef	0.01
5	(±)-9-(2-cyclopenten-1-yl)nonan-1-ol acetate	0d	31d	0.03
(S)-5	(-)-(S)-9-(2-cyclopenten-1-yl)nonan-1-ol acetate	0d	35d	0.30

^a 100 ng compound or mixture on filter paper. Twenty replicates/treatment. Means followed by the same letter are not significantly different according to

^b T-tests (LSD), $P = 0.05$.

^c Mean percentage of males exhibiting clasper extension and copulatory strikes.

^d Data from Bestmann et al. (1980).

TABLE 2. RESPONSE OF 3 MALE ECB PHEROMONAL TYPES TO MIXTURES OF 11-TETRADECEN-1-OL ACETATES AND ANALOGS IN FLIGHT TUNNEL

Compound	Amount (ng)	Treatment	Upwind flight (%)
<i>ZZ</i> males ($N = 43$) 100 ng/compound			
1 + 2		11-tetradecen-1-ol acetate (97:3, <i>Z:E</i>)	85a
3		(<i>Z</i>)-11-tridecen-1-ol acetate	59ab
7		(<i>Z</i>)-13-methyl-11-tetradecen-1-ol acetate	56ab
9		(<i>Z</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	53b
11		(<i>Z</i>)-13,13-dimethyl-11-tetradecen-1-ol acetate	5c
<i>EE</i> males ($N = 39$) 100 ng/compound			
1 + 2		11-tetradecen-1-ol acetate (3:97, <i>Z:E</i>)	77a
8		(<i>E</i>)-13-methyl-11-tetradecen-1-ol acetate	56a
4		(<i>E</i>)-11-tridecen-1-ol acetate	31b
12		(<i>E</i>)-13,13-dimethyl-11-tetradecen-1-ol acetate	12bc
10		(<i>E</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	2c
<i>ZE</i> males test 1 ($N = 39$)			
1 + 2	100	11-tetradecen-1-ol acetate (40:60, <i>Z:E</i>)	92a
9	200	(<i>Z</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	87a
9 + 2	200	(<i>Z</i>)-12-cyclopropyl-11-dodecen-1-ol acetate +	
	60	(<i>E</i>)-11-tetradecen-1-ol acetate	77ab
1	40	(<i>Z</i>)-11-tetradecen-1-ol acetate	64ab
10 + 1	300	(<i>E</i>)-12-cyclopropyl-11-dodecen-1-ol acetate +	
	40	(<i>Z</i>)-11-tetradecen-1-ol acetate	48b
10	300	(<i>E</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	13c
2	60	(<i>E</i>)-11-tetradecen-1-ol acetate	0c
<i>ZE</i> males test 2 ($N = 43$)			
1 + 2	100	11-tetradecen-1-ol acetate (40:60, <i>Z:E</i>)	88a
7	40	(<i>Z</i>)-13-methyl-11-tetradecen-1-ol acetate	81a
8	60	(<i>E</i>)-13-methyl-11-tetradecen-1-ol acetate	47b
7 + 8	100	13-methyl-11-tetradecen-1-ol acetate (40:60, <i>Z:E</i>)	91a

flight response relative to the pheromone alone. When ECB moths in a cage were preexposed to analog **10** alone for 3 min and then exposed to the mixture, a similar reduction in upwind flight response was observed. Exposure of *EE* males to a mixture of the natural pheromone (3:97, *Z:E*) and **12** resulted in no reduction of upwind flight response, nor was any reduction in upwind flight response observed when the moths were preexposed to analog **12** and then exposed to the mixture.

Response of ZZ and EE Males to 11-Tetradecen-1-ol Acetate Isomers. Results (Table 4) of exposing *ZZ* males to pure (*Z*)-11-tetradecen-1-ol-acetate (**1**) or the 97:3 natural pheromone blend of **1** or **2** at different doses showed

TABLE 3. COMPARISON OF RESPONSES OF *EE*-TYPE MALE ECB TO MIXTURES OF 11-TETRADECEN-1-OL ACETATES AND ANALOGS WITH AND WITHOUT PREEXPOSURE TO SAME ANALOGS IN FLIGHT TUNNEL

Compound	Treatment	Upwind flight (%)
Test 1 (<i>N</i> = 30)		
1 + 2	100 ng 11-tetradecen-1-ol acetate (3:97, <i>Z</i> : <i>E</i>)	77
1 + 2 + 10	100 ng 11-tetradecen-1-ol acetate (3:97, <i>Z</i> : <i>E</i>) + 3 μg (<i>E</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	13
10; 1 + 2 + 10	3 μg (<i>E</i>)-12-cyclopropyl-11-dodecen-1-ol acetate ^a 100 ng 11-tetradecen-1-ol acetate (3:97, <i>Z</i> : <i>E</i>) + 3 μg (<i>E</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	27
Test 2 (<i>N</i> = 20)		
1 + 2	100 ng 11-tetradecen-1-ol acetate (3:97, <i>Z</i> : <i>E</i>)	90
1 + 2 + 12	100 ng 11-tetradecen-1-ol acetate (3:97, <i>Z</i> : <i>E</i>) + 3 μg (<i>E</i>)-13,13-dimethyl-11-tetradecen-1-ol acetate	70
12; 1 + 2 + 12	3 μg (<i>E</i>)-13,13-dimethyl-11-tetradecen-1-ol acetate ^a 100 ng 11-tetradecen-1-ol acetate (3:97, <i>Z</i> : <i>E</i>) + 3 μg (<i>E</i>)-13,13-dimethyl-11-tetradecen-1-ol acetate	80

^aMoths were preexposed to pure analog in a cage for 3 min, then exposed to a mixture of natural pheromone and analog.

that in the flight tunnel males could not discriminate between treatments. The same results were obtained when *EE* males were exposed to different doses of pure (*E*)-11-tetradecen-1-ol acetate (2) or to the 3:97 natural pheromone blend of 1 and 2 or to a 50:50 blend of the same compounds.

TABLE 4. RESPONSE OF TWO PHEROMONAL TYPES OF MALE EUROPEAN CORN BORER IN FLIGHT TUNNEL TO 11-TETRADECEN-1-OL ACETATE ISOMERS EVAPORATED FROM FILTER PAPER AND FROM RUBBER SEPTA

11-Tetradecen-1-ol acetate, <i>Z</i> : <i>E</i>	Dose	Male response (No. upwind/No. males tested)
<i>ZZ</i> males		
100:0	100 ng/paper	11/15
97:3	100 ng/paper	11/15
100:0	30 μg/septum	16/24
97:3	30 μg/septum	21/24
<i>EE</i> males		
0:100	100 ng/paper	11/15
3:97	100 ng/paper	12/15
3:97	100 μg/septum	9/10
1:1	100 μg/septum	8/10

TABLE 5. CAPTURE OF MALE EUROPEAN CORN BORER IN TRAPS BAITED WITH SEX PHEROMONE AND PHEROMONAL ANALOGS

Compound	Treatment compound (<i>Z:E</i>) ^a	\bar{X} male/trap/night ^b
3	(<i>Z</i>)-11-tridecen-1-ol acetate	2.3de
4	(<i>E</i>)-11-tridecen-1-ol acetate	1.3de
11 + 12	13,13-dimethyl-11-tetradecen-1-ol acetate (97:3)	0.2e
12	(<i>E</i>)-13,13-dimethyl-11-tetradecen-1-ol acetate	1.0e
1 + 2	11-tetradecen-1-ol acetate (3:97)	16.6b
1 + 2	11-tetradecen-1-ol acetate (97:3)	69.2a
9	(<i>Z</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	8.3c
9 + 10	12-cyclopropyl-11-dodecen-1-ol acetate (3:97)	5.4cd
7	(<i>Z</i>)-13-methyl-11-tetradecen-1-ol acetate	1.0de
8	(<i>E</i>)-13-methyl-11-tetradecen-1-ol acetate	1.0de

^aTraps baited with rubber septa treated with 100 μ g of indicated compound or mixture. Replication over 12 consecutive nights in the vicinity of Beltsville, Maryland, July 23–August 3, 1986.

^bMeans followed by the same letter are not significantly different. Raw data were transformed to square root for significance tests.

Field Capture of ECB Males in Traps Baited with Synthetic Sex Pheromone and with Pheromonal Analogs. Among the analogs tested (Table 5), only the (*Z*)-cyclopropyl analog (**9**), showed a modicum of activity. The effectiveness of **9** was not enhanced by the addition of 3% of the *E* compound **10**. However, the effectiveness of **9** was enhanced by the addition of **2** (Table 6). This series of trials also confirmed the very low order of activity of pure **1** under field conditions.

TABLE 6. INFLUENCE OF GEOMETRICAL COMPOSITION UPON EUROPEAN CORN BORER MALE TRAP CAPTURE AND EFFECT OF USING A TRACE OF OPPOSITE GEOMETRICAL PHEROMONE COMPONENT IN COMBINATION WITH PHEROMONAL ANALOG

Compound	Amount (μ g)	Treatment compound/rubber septum	\bar{X} male/trap/night ^a
1 + 2	100	11-tetradecen-1-ol acetate (97:3, <i>Z:E</i>)	43.3a
9	248.3	(<i>Z</i>)-12-cyclopropyl-11-dodecen-1-ol acetate	5.2c
9 + 2	248.3	(<i>Z</i>)-12-cyclopropyl-11-dodecen-1-ol acetate +	
	17.5	(<i>E</i>)-11-tetradecen-1-ol acetate	17.9b
2	17.5	(<i>E</i>)-11-tetradecen-1-ol acetate	1.8c
1	100	(<i>Z</i>)-11-tetradecen-1-ol acetate	1.8c

^aMeans followed by the same letter are not significantly different. The test was conducted over four consecutive nights, September 4–7, 1987, with three replicates/night.

TABLE 7. RESPONSE OF REDBANDED LEAFROLLER (*Argyrotaenia velutinana*) MALES IN FLIGHT TUNNEL TO 7-COMPONENT SYNTHETIC PHEROMONAL MIXTURE (Bjostad et al., 1985), PHEROMONAL ANALOG ALONE, AND ANALOGS SUBSTITUTED FOR (Z)- and (E)-11-TETRADECEN-1-OL ACETATE IN MIXTURE

Compound No.	Amount (μ g)	Treatment ^a	Flight Tunnel % Upwind flight to source
Test 1 (N = 15)			
1 + 2	100	(Z)-11-tetradecen-1-ol acetate +	60
	9	(E)-11-tetradecen-1-ol acetate + 5 compounds (natural)	
7 + 8	100	(Z)-13-methyl-11-tetradecen-1-ol acetate +	7
	9	(E)-13-methyl-11-tetradecen-1-ol acetate + 5 compounds	
Test 2 (N = 10)			
1 + 2		Natural	90
9 + 10	248	(Z)-12-cyclopropyl-11-dodecen-1-ol acetate +	0
	23	(E)-12-cyclopropyl-11-dodecen-1-ol acetate + 5 compounds	
Test 3 (N = 15)			
1 + 2		Natural	87
3 + 4	100	(Z)-11-tridecen-1-ol acetate +	53
	9	(E)-11-tridecen-1-ol acetate + 5 compounds	
3 + 4	100	(Z)-11-tridecen-1-ol acetate +	73
	9	(E)-11-tridecen-1-ol acetate	

^aCompounds applied to rubber septa.

Response of RBLR Male Moths to Pheromonal Analogs in Flight Tunnel.

When compounds **7** and **8** were substituted for a 100:9 mixture of **1** and **2** in the natural pheromone, only a weak upwind flight response was elicited, and when **9** and **10** were substituted for **1** and **2**, no response was observed (Table 7). Substitution of **1** and **2** with **3** and **4** in the natural pheromone or a mixture of **3** and **4** alone evoked high upwind flight activity.

DISCUSSION

At the outset of this investigation we were intrigued by the report (Bestmann et al., 1980) that (*R*)-**6** was 33 times more EAG active than (*S*)-**5**, which was reported by Chapman et al. (1978a,b) to be their most active analog in an ECB sex-stimulation bioassay. We anticipated that the former compound might

also be proportionately more active than the latter in the whole organism behavioral bioassay. The results of the flight-tunnel and sex-stimulation assays (Table 1), when compared with the EAG data presented by Bestmann et al. (1980), show that even though the relative EAG activities within each set of compounds were consistent with the results observed in the male sex stimulation assay, the relative biological activity of the compounds in the sex stimulation assay was completely different: (*S*)-**5** was twice as active as (*R*)-**6**. It is evident in this case that EAG is not necessarily a reliable predictor of the behavior inducing potency of a compound.

Data in Table 1 also show that although the Chapman and Bestmann compounds evoked sex stimulation responses, they were not adequate to cause upwind flight in the tunnel. Only the first five treatments in Table 1 induced ECB males to fly to the source and, of these, only blends of **3** and **4** quantitatively mimicked the natural pheromone. Furthermore, from previous work (Klun and Robinson 1972), 11-dodecen-1-ol acetate is known to be completely inactive. It is apparent from the results that for an analog to evoke an upwind flight response, it must have a flexible structure of either 13 or 14 carbons in length. It should be noted that **7** falls within that criterion and was active in the flight tunnel while **5** and **6** were not. Flight-tunnel test results with the three types of ECB males (Table 2) showed that cyclopropyl analog **9**, which may be formally considered a 14-carbon analog, possessed considerable activity in the flight tunnel, while the (*E*)- and (*Z*)-cyclobutyl analogs **13** and **14**, which may be considered 15-carbon analogs, were completely inactive in all assays. In contrast, (*E*)- and (*Z*)-*tert*-butyl analogs **11** and **12**, which are of 14-carbon chain length, showed only marginal activity. Thus, results show that biological activity is not only dependent upon a 13- or 14-carbon chain length, but is also impacted by the bulk at carbon 13 and/or the absence of a proton at that position.

These general structural requirements held true in our flight-tunnel tests against *ZZ* type males. However, the results of tests with analog **10** against *EE* males were unexpected. The activity of all *E* analogs against *EE* males paralleled the activity of the *Z* analogs against the *ZZ* males with the exception of **10**; it was biologically inactive against the *EE* males. Similarly, **10** was inactive against the *ZE* males while **9** was active. To account for the surprising lack of biological activity of **10** against *ZE* and *EE* males, we considered the possibility that (*E*)-cyclopropyl analog **10** might assume a minimum energy conformation that was very different from the conformations assumed by the other active *E* compounds, and that the active *Z* compounds, including the (*Z*)-cyclopropyl analog **9**, might assume similar minimum energy conformations. Calculations showed that this was the case; however, the energy required for **10** to assume a conformation resembling the minimum energy conformation of the active *E* compounds was only ca. 1 kcal/mol higher than its intrinsic minimum energy

conformation. This energy difference was too small to prevent (*E*)-cyclopropyl analog molecules from assuming a conformation that resembles the active *E* compounds.

The inactivity of **10** most likely is related to intrinsic differences of the *E* and *Z* receptors and to the fact that **9** and **10** interact differently with these receptors. Normal binding is thought to involve a three-pronged interaction of the pheromone molecule with the acceptor by hydrogen bonding and/or electrostatic interaction to the functional group, electrostatic interaction with the double bond, and much weaker dispersion forces responsible for binding to the terminal alkyl group (Liljefors et al., 1985). Dispersion forces are considered to be very weak, and they fall off rapidly with the distance between interacting centers. Thus, as Liljefors et al. (1985) have pointed out, a close fit between the terminal alkyl group and receptor site is extremely important to make it possible for this interaction to take place. We speculate that the cyclopropyl group of analog **10**, as a consequence of its electron-donating properties, may bind to an electrophilic site of the *E* receptor. This electrophilic site may not be present in the *Z* receptor. The forces that bind **10** to the (*E*)-11-tetradecen-1-ol acetate receptor site probably involve electrostatic interactions also at the cyclopropyl moiety, and these forces may be stronger than those that bind the other biologically active compounds at their terminus. However, because binding takes place in a manner that is inappropriate for transmission of a pheromonal signal, no behavioral activity is observed.

To test the hypothesis that **2** and **10** interact with the same receptor, a competition experiment consisting of three treatments was carried out (Table 3). In one treatment males were exposed to pheromone alone, and in another males were exposed to a mixture of pheromone and **10**. In a third treatment males were preexposed to **10** and then exposed to the mixture. The purpose of the preexposure was to cause sensory adaptation to **10**. If the binding site for **10** were different from the pheromone receptor site, one would expect the response of males subsequently exposed to pheromone containing **10** to be the same as the response elicited by pheromone alone; males would perceive and respond to the mixture as if only pheromone was present. Results showed that response to the mixture, with or without preexposure to **10**, was significantly reduced relative to the response to pheromone alone. Therefore, by inference, **10** and **2** must interact with the same receptor.

In a corollary experiment, the behavioral effects of a mixture of the natural pheromone and (*E*)-*tert*-butyl analog **12**, with and without preexposure to **12**, was compared with the activity of the natural pheromone alone. Results (Table 3) showed that **12** did not interfere with perception of **2**. Thus, although **10** and **12** each fail to induce a male behavioral response, the physical basis for the inactivity of each is different. The biological inactivity of **10** is related to an inappropriate interaction with the receptor. On the other hand, the inactivity of

12 is the consequence of the steric bulk of the *tert*-butyl group, which prevents it from interacting with the pheromone receptor.

An additional factor involved in the dissimilar biological activities of **9** and **10** may also be associated with differences in structure between the *E* and *Z* receptors in the pheromonal types. Flight-tunnel results of tests with *ZE* type males (Table 2) showed not only that **10** was inactive, but that **2**, which constitutes the major component of the natural hybrid pheromone, also proved inactive when tested by itself, while **1**, when tested alone, was active. Nevertheless, **2** was active when tested singly in the flight tunnel against the *EE* type males (Table 4).

Because the pheromones of all three ECB types are known to be mixtures of **1** and **2**, it was of interest to learn what effect substitution of one of the components of the natural pheromones by an analog of identical geometrical configuration would have on male responses in the flight tunnel. Preliminary to these assays, we tested the biological activities of pure **1** and the natural 97:3 blend of **1** and **2** in the *ZZ* type, and pure **2** and the natural 3:97 blend of **1** and **2** in the *EE* type. As shown in Table 4, under our flight-tunnel conditions, both types of males were unable to differentiate among these treatments. Therefore, undertaking of these substitution experiments was obviated. It should be noted, however, that under field conditions there is no doubt that geometrically pure **1** exhibited only negligible activity and that a blend of 97:3 of **1** and **2** is substantially more effective in trapping male ECB (Table 6).

The most conclusive test for biological activity of a sex pheromone analog is a field test, because biological screening at this level most effectively shows how closely the analog mimics the natural product on all behavioral levels. For practical purposes all analogs, except for marginal activity of analogs **9** and **10**, were inactive in field trapping tests (Table 5). The activity shown for the 3:97 mixture of **9** and **10** may be attributed to the presence of **9**, and the relatively large number of ECB captured by this treatment would be due to the preponderance of *ZZ*-type ECB in the Beltsville, Maryland, area (Klun and Huettel, 1988). A second field trial (Table 6) conducted with analog **9** showed that in their natural habitat, the insects discriminate between probes. Thus, a mixture of **9** and **2** in the appropriate ratio to mimic the natural pheromone blend, captured 3.5 times as many insects as **9** did by itself.

Another objective of our study was to examine interspecific relationships of pheromone perception. The RBLR uses as its pheromone a mixture of seven acetates, of which **1** and **2** are the major components (Bjostad et al., 1985). It had been shown previously (Chapman et al., 1978a,b) that in sex stimulation bioassays, (*R*)-**5**, (*S*)-**5**, and racemic **5** elicited strong male responses from RBLR. Sex stimulation responses were also observed when RBLR males were exposed to **7** and **8**. However, no sex stimulation behavior was observed when RBLR males were exposed to **9** and **10**. In flight-tunnel bioassays (Table 7) of

mixtures of **7** and **8**, only ca. 7% of RBLR males responded by flying to the source, and no response in the flight tunnel was observed to mixtures of **9** and **10**. When these treatments were administered simultaneously with the natural pheromone, they neither enhanced nor inhibited the effect of the latter. These results suggest that the RBLR and the ECB have pheromone receptor systems of different structures, a fact that was already indicated by Chapman et al. (1978a,b).

In summary, we have shown that the ECB pheromone receptor system imposes severe structural limitations on candidate mediators of sexual signals. Among the analogs discussed, where only changes in the terminal alkyl group were made, chain length and bulk are closely circumscribed. The ECB males display a hierarchy of sexual responses, and the effects of modification of pheromone structure can be monitored at the various behavioral levels by the use of sex-stimulation bioassays, flight-tunnel assays, or field trials. Each of these tests imposes progressively higher strictures on the permissible modification of chemical structure. The dissimilarity of impact of the (*Z*)- and (*E*)-cyclopropyl analogs, **9** and **10**, on the chemoreception systems of the *EE* and *ZZ* types of the ECB may be a consequence of differences in structures of the pheromone receptor site in European corn borer pheromonal types. Finally, we have shown that even though the ECB and RBLR use the same pheromonal compounds, the nature of the acceptor systems for (*E*)- and (*Z*)-11-tetradecen-1-ol acetate are different.

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PHYTOTOXICITY OF WATER-SOLUBLE SUBSTANCES FROM ALFALFA AND BARLEY SOIL EXTRACTS ON FOUR CROP SPECIES¹

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Abstract—Problems associated with continuously planting alfalfa (*Medicago sativa* L.) or seeding to thicken depleted alfalfa stands may be due to autotoxicity, an intraspecific form of allelopathy. A bioassay approach was utilized to characterize the specificity and chemical nature of phytotoxins in extracts of alfalfa soils as compared to fallow soil or soil where a cereal was the previous crop. In germination chamber experiments, water-soluble substances present in methanol extracts of soil cropped to alfalfa or barley (*Hordeum vulgare* L.) decreased seedling root length of alfalfa L-720, winter wheat (*Triticum aestivum* L. Nugaines) and radish (*Raphanus sativa* L. Crimson Giant). Five days after germination, seedling dry weights of alfalfa and radish in alfalfa soil extracts were lower compared to wheat or red clover (*Trifolium pratense* L. Kenland). Growth of red clover was not significantly reduced by soil extracts from cropped soil. Extracts of crop residue screened from soil cropped to alfalfa or barley significantly reduced seedling root length; extracts of alfalfa residue caused a greater inhibition of seedling dry weight than extracts of barley residue. A phytotoxic, unidentified substance present in extracts of crop residue screened from alfalfa soil, which inhibited seedling root length of alfalfa, was isolated by thin-layer chromatography (TLC). Residues from a soil cropped continuously to alfalfa for 10 years had the greatest phytotoxic activity.

Key Words—Allelopathy, autotoxicity, *Medicago sativa* L., germination, seedling establishment, alfalfa, crop residues, phenolic acids.

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INTRODUCTION

Many crop and weed plants contain phytotoxic substances which, when released into the environment, can cause reduced plant growth (Rice, 1984). Patrick (1971) found the majority of phytotoxic material present under field conditions is confined to the loci of decomposing residue rather than the surrounding soil. Guenzi and McCalla (1966) reported ferulic and *p*-coumaric acids were among the inhibitors present in the mature crop residues of corn (*Zea mays* L.), wheat, sorghum (*Sorghum bicolor* L., Moench.), and oat (*Avena sativa* L.) and in the soil (0–7.5 cm depth) under stubble-mulched plots (Guenzi and McCalla, 1966). Guenzi et al. (1964) found alfalfa phytotoxicity interacted with growth stage but not with cultivars, and water extracts of immature alfalfa forage had the greatest phytotoxic effects on corn seedlings in laboratory studies.

A water-soluble phytotoxin derived from alfalfa residues has been implicated in the problems associated with poor stand establishment on soils continuously cropped to alfalfa, giving rise to the term "alfalfa-autotoxicity" (McElgunn and Heinrichs, 1970; Miller, 1983). Webster et al. (1967) observed a larger yield increase from sterilized soil and soil fumigation by Vapam (sodium *N*-methyl dithiocarbamate) application than fertilization of two "alfalfa-toxic soils." They concluded improved growth by sterilization indicates the alfalfa autotoxin is of biological origin. Jensen (1984) reported alfalfa seedlings were smaller when grown in alfalfa soil compared to fallow soil, even though both soils were steam sterilized. Ground foliage or roots of alfalfa added to both soils significantly inhibited alfalfa seedling size and the number of plants established. Similarly, Ells and McSay (1984) reported that adding alfalfa foliage to soil inhibits germination of cucumber (*Cucumis sativus* L.).

Research in Illinois suggests alfalfa is difficult to establish, and yields are reduced when alfalfa is planted continuously (Klein and Miller, 1980). Kehr et al. (1983) reported average yields over a three-year period were 34% higher on land not previously cropped to alfalfa compared to land reseeded to alfalfa. They concluded autotoxicity was involved in lower yields of continuous alfalfa if moisture and pathogen conditions are included. Miller (1983) reported a significantly lower seedling population and second-year dry matter yield with a continuous cropping of alfalfa even though P and K deficiencies were corrected and seeds were treated with Captan (*N*-trichloromethyl thiotetrahydro-phthalimide) to protect seedlings against fungal attack.

Tesar (1984) at Michigan obtained successful stands of alfalfa following alfalfa when seeding in spring or summer after at least two weeks following spraying an old stand with glyphosate. However, he prefers spring seeding on a prepared seed-bed after fall plowing of alfalfa. Improper management does not appear to be responsible for difficulties in reestablishment in many instances.

Red clover and alsike clover (*Trifolium hybridum* L.) are not affected by the unknown phytotoxin (Webster et al., 1967; Jensen, 1984).

The objectives of this research were to determine: (1) the ecological source of the phytotoxic material from soil or plant residues, (2) the degree of auto-toxicity in soils where alfalfa had been grown for various periods of time, and (3) the chemical nature of the phytotoxins.

METHODS AND MATERIALS

Sampling. Approximately 75 liters of soil were collected by soil auger (0–15 cm depth) from each of five fields in the fall of 1984 subsequent to the last harvest date at each location. The soils collected were from a one-year alfalfa stand, an eight-year alfalfa stand, a 10-year alfalfa stand, a spring barley stand recently turned under, and from ground fallowed for four years, hereinafter referred to as 1-MESA, 8-MESA, 10-MESA, 1-HOVU, and fallow soil, respectively.

Subsamples of each soil (15 liters) were separated into three fractions: (1) soil as taken from site, hereinafter referred to as whole soil, (2) soil screened to pass a 1.2-mm sieve, and (3) plant residues from screened soil, i.e., plant residues not passing a 1.2-mm sieve. The fallow soil treatment consisted of unscreened whole soil. Residue samples were characterized as having 0.3- to 3.0-cm-long pieces of stem and root; adhering soil particles were not removed. Soil and residue samples were stored at -10°C .

Extraction. Extracts were prepared prior to each trial. Following the method of Guenzi and McCalla (1966), the amount of soil used is expressed on an oven-dry basis, but extractions were made at approximately the same moisture content as existed at the time of sampling. A 250-g sample of oven-dry soil (whole or screened) was extracted with anhydrous methanol (1:1, w/v). Barley or alfalfa residues (16.6 g fresh weight) were mixed in a blender with 250 ml anhydrous methanol. Soil and residue samples were extracted separately for 2 hr on a reciprocating shaker and left to stand for 12 hr. Extracts were filtered through Whatman No. 4 filter paper, reduced to dryness in a rotary flask evaporator at 40°C , and the dried residue resuspended in 50 ml autoclaved water. Extracts were stored at 4°C overnight in sterile tubes.

Bioassays of Soil and Residue Extracts. Sterile Petri dishes ($9 \times 9 \times 1$ cm) were fitted with blotter paper moistened with 2 ml autoclaved, distilled water. Fifty surface-sterilized seeds of alfalfa or red clover and 25 seeds of wheat or radish were established in these dishes. Extracts (5 ml) of soil or crop residue were delivered aseptically, with an additional 5 ml added after 24 hr. The germination chamber for the germination and seedling development tests

was held at 20°C with relative humidity near saturation. Electrical conductivity (EC) and pH were determined on the portion of extract (10 ml) not used for a bioassay in each trial.

Each Petri dish, with a ruler alongside as a reference scale, was photographed 48 hr after planting. Fifteen seedlings in the photograph were measured to the nearest 0.5 mm. Dishes were returned to the germinator for an additional three days. Seedlings received 12 hr of fluorescent light (17.5 W/m²) 108 hr after planting. Five days after initiating the trial, seedlings were counted and dried at 60°C for 48 hr and average dry weight per seedling determined (AOSA, 1983).

Statistical design was a split-split plot with soils as main plots, soil or crop residue extracts as subplots, and species as sub-subplots. Seedling root lengths and average seedling dry weights of the four bioassay species were determined in four replications. Data, expressed as a percent of the fallow soil extract, were subjected to analysis of variance (SAS, 1984), and treatment means were compared by Duncan's new multiple-range test at the 5% level of significance (Steele and Torrie, 1980).

Chromatographic Biosassay. An aliquot (7 ml), obtained from a pooled sample (about 30 ml) of whole soil or crop residue extracts, was reduced to dryness in a rotary flask evaporator at 40°C. The dried residue was resuspended in 2 ml anhydrous methanol. Spotting of TLC plates (0.25 mm thick, Silicagel G, F254 nm) was done using 40 µl of barley or alfalfa residue extracts (1.16 g residue/ml) and 40 µl of whole soil extracts (17.5 g soil/ml). Since most phytotoxic substances were thought to be phenolic in nature, 20 µl of each of 10 phenolic compounds (1 mg/ml methanol) were developed simultaneously. Compounds separated by one-dimensional TLC in a solvent system of *n*-butanol-acetic acid-water (4:1:5, v/v) were observed by fluorescence quenching (254 nm).

Based on the methods of Liebl and Worksham (1983), 200 µl of an extract were spotted in each of six lanes on 2.0-mm-thick TLC plates. Six identical spots of an isolated compound observed under UV light were scraped from a TLC plate, eluted with 10 ml acetone, and filtered through Whatman No. 42 filter paper. Acetone was evaporated under N₂ and the extract resuspended in 10 ml autoclaved, distilled water. Forty surface-sterilized seeds of alfalfa were soaked in an extract solution for 4 hr at room temperature. Ten seeds were removed from a flask and transferred to a sterile Petri dish (9 × 9 × 1 cm) fitted with germination paper which then received 1/4 of the remaining volume of bioassay solution remaining after 4 hr of imbibition. The radicle lengths of 10 alfalfa seedlings were measured in four replications after incubation for 72 hr. Statistical design was a two-way factorial arrangement with *R_f* values as treatment levels in bioassays of residue extracts from the four cropped soils.

Data were subjected to analysis of variance and means separated by the least significant difference (LSD) test at the 5% level of significance (Steele and Torrie, 1980).

RESULTS

Germination of alfalfa, red clover, radish, or wheat was not significantly inhibited by the various soil extracts. In terms of seedling root length and dry weight, individual species responded differently to extracts from soils with different cropping histories (Tables 1 and 3). Root length of wheat was inhibited significantly more than the other species by 10-MESA soil extracts, and root length of red clover was inhibited the least (Table 1). The extracts from 8-MESA and 10-MESA soils were most inhibitory to wheat. Root lengths of alfalfa were reduced more than those of red clover, but responded similarly to extracts of soils with different cropping histories. Extracts of 1-HOVU significantly inhibited root length of radish more than extracts from soils cropped to alfalfa (Table 1).

Extracts from screened soil increased root length of all species compared to extracts from fallow soil except for wheat (Table 2). Alfalfa and wheat root lengths were inhibited the most by residue extracts, while radish was inhibited to a lesser degree. Roots of alfalfa were significantly longer when grown in extracts from screened soil than either whole soil or residue extracts. This indicates the toxic properties of soil to seedling root growth of alfalfa is related to the residue fraction of the soil. Red clover root lengths were not significantly different when grown in extracts from the three soil treatments. This agrees with

TABLE 1. PERCENT CHANGE IN SEEDLING ROOT LENGTH OF FOUR SPECIES GROWN IN EXTRACTS OF FOUR SOILS WITH DIFFERENT CROPPING HISTORIES COMPARED TO GROWTH IN EXTRACTS FROM FALLOW SOIL

Species	Root length (% change)			
	1-MESA	8-MESA	10-MESA	1-HOVU
Red clover	+0.86d ^a	-1.25d	+1.38d	-1.88cd
Alfalfa	-3.55cd	-6.58bcd	-10.10bcd	-14.78bc
Wheat	-8.13bcd	-19.72b	-36.68a	-6.02bcd
Radish	+2.83d	+1.32d	+3.11d	-17.61bc

^a Any means followed by the same letter are not significantly different at the 5% level of probability using Duncan's new multiple-range test.

TABLE 2. PERCENT CHANGE IN SEEDLING ROOT LENGTH OF FOUR SPECIES GROWN IN EXTRACTS OF WHOLE SOIL, SCREENED SOIL, AND SOIL RESIDUE COMPARED TO GROWTH IN EXTRACTS FROM FALLOW SOIL

Species	Root length (% change)		
	Whole	Screened	Residue
Red Clover	-0.29cde ^a	+2.89cde	-3.27cd
Alfalfa	-5.78c	+12.17e	-32.65a
Wheat	-11.53bc	-5.53c	-35.84a
Radish	-0.07de	+10.46e	-18.14b

^a Any means followed by the same letter are not significantly different at the 5% level of probability using Duncan's new multiple-range test.

our field observations (Jensen, 1984). The interaction of soils \times soil treatment \times species was not significant at the 5% level of probability.

Seedling dry weights of alfalfa and radish were reduced significantly more by 1-MESA or 8-MESA soil extracts than were red clover or wheat (Table 3). Seedling dry weight of wheat was reduced when grown in extracts from 8-MESA and 10-MESA soils compared to extracts from an unscreened fallow soil. Alfalfa seedling weights were reduced most when grown in alfalfa soil extracts and least when grown in extracts from a 1-HOVU soil. Dry weights of radish seedlings were affected in a similar manner as alfalfa when grown in extracts from soil with different cropping histories.

Average dry weights of seedlings grown in extracts from soil residue were significantly reduced more than those grown in extracts from whole or screened

TABLE 3. PERCENT CHANGE IN SEEDLING DRY WEIGHT OF FOUR SPECIES GROWN IN AQUEOUS EXTRACTS OF FOUR SOILS WITH DIFFERENT CROPPING HISTORIES COMPARED TO GROWTH IN EXTRACTS FROM FALLOW SOIL

Species	Dry Weight (% change)			
	1-MESA	8-MESA	10-MESA	1-HOVU
Red clover	-0.62cd*	+2.06d	-2.54bcd	-2.47bcd
Alfalfa	-10.30ab	-11.75a	-8.82bc	-5.51bcd
Wheat	+7.31d	-1.35cd	-10.57ab	+1.74d
Radish	-11.35ab	-13.45a	-10.58ab	-5.34bcd

^a Any means followed by the same letter are not significantly different at the 5% level of probability using Duncan's new multiple-range test.

soil (Table 4). Seedling weight reductions were intermediate in the seedlings grown in extracts from screened soil and least when grown in extracts from whole soil. The interaction of soil treatment \times species was not significant and neither was the interaction of soils \times soil treatment \times species.

The results of these trials suggest seedling vigor of alfalfa was inhibited by extracts of 1-, 8-, and 10-MESA over the five-day period (Tables 2 and 3). In addition, the seedling root length and dry weight of radish were inhibited to the same extent as alfalfa. Thus, seedling vigor of radish may be useful as an indicator for bioassays involving phytotoxins extracted from alfalfa residues.

Extracts of residue screened from the barley, 10-, 1-, and 8-year alfalfa soils, and fallow soil had pH values of 5.0, 4.6, 5.5, 6.1, and 6.8, respectively. The presence of dissolved salts did not interact with reduced seedling vigor. Inhibitory extracts had EC values less than fallow soil, which had an average EC of 3.48 dS/m.

Water-soluble substances in extracts of crop residues concentrated at $R_f = 0.78$ significantly inhibited alfalfa root length when compared to substances at $R_f = 0.87$. These substances present in 10- or 1-MESA residue significantly inhibited root length of alfalfa seedlings (Table 5). The substances with an $R_f = 0.78$ in 10-MESA residue caused an irreversible inhibition of germination. When these alfalfa seeds were washed with autoclaved water and placed back in the germinator, they did not germinate. A relatively high germination in solutions of 1-MESA residue ($R_f = 0.78$) was not accompanied by good seedling growth. Toxic substances caused symptoms other than inhibition of root growth. These were a swelling of root tips, curling of the root axis, discoloration, and lack of root hairs.

TABLE 4. PERCENT CHANGE IN DRY WEIGHT OF FOUR SPECIES GROWN IN EXTRACTS OF WHOLE SOIL, SCREENED SOIL, AND SOIL RESIDUE COMPARED TO GROWTH IN EXTRACTS FROM FALLOW SOIL

Species	Dry weight (% change)			
	Whole	Screened	Residue	Average
Red Clover	+0.13ns	+0.61	-3.38	-0.89b ^a
Alfalfa	-8.89	-7.64	-10.82	-9.09a
Wheat	+5.71	-1.85	-6.02	-0.72b
Radish	-8.07	-7.83	-14.61	-10.18a
Average	-2.78b	-4.18b	-8.71a	

^aAny means, within a group, followed by the same letter are not significantly different at the 5% level of probability using Duncan's new multiple-range test. ns: The interaction of soil treatment \times species was not significant.

TABLE 5. R_f VALUES AND ROOT LENGTH OF ALFALFA SEEDLINGS IN SUBSTANCES ISOLATED BY TLC FROM CROP RESIDUES^a

Origin of residue extracts	Root length (mm)	
	$R_f = 0.78$	$R_f = 0.87$
1-HOVU	10.3	11.0
1-MESA	7.7	11.5
8-MESA	13.9	11.1
10-MESA	1.0	10.7
Distilled water	14.3	14.3

^aLSD (0.05) = 1.5; CV = 10%.

A possible explanation for growth inhibition is the presence of phenolic substances, since the inhibitory band developed with an R_f similar to *p*-coumaric acid ($R_f = 0.74$) and *trans*-cinnamic acid ($R_f = 0.75$) and similarly quenched fluorescence of the silica gel phosphor. The fluorescence emission of the inhibitory region and ferulic acid ($R_f = 0.67$) were similar, but differing mobilities indicate toxicity from ferulic acid is unlikely.

Further examination of this inhibitory band ($R_f = 0.78$) from 1-, 8-, and 10-MESA and 1-HOVU crop residues was performed by elution with acetone and concentration to 1 ml. Methylation of the eluted band followed by gas chromatographic analysis did not reveal significant amounts of either cinnamic or *p*-coumaric acid. Methylation of the crude methanol extract of 10-MESA residue followed by gas chromatographic analysis similarly did not reveal any high concentration of compounds that could be responsible for the observed phytotoxicity. Although concentrations of about 10^{-4} M were detected with these methods, some phenolic acids, in particular some of the monomeric phenolic acids, are toxic to plants and to certain microorganisms at concentrations greater than about 10^{-5} M to 10^{-4} M in solution (Whitehead et al., 1982).

DISCUSSION

In dealing with production of phytotoxins in soil, methods for detection should reflect the ecological condition of the soil as encountered by the plant. In this study, the residue extracts represented 6.67% (1:15 w/w) of the soil mass on a fresh weight basis. Patrick (1971) found the severity of injury to seedling roots was related to the amount of plant residue in the immediate vicinity of the root. He reported toxic substances were only extracted from clumps of residue in which the percentage of soil did not exceed 80% on a fresh weight

basis. Under field conditions, phytotoxins were most abundant in the early stages of decomposition of plant residues during periods of water saturation (Patrick, 1971).

Results from this bioassay suggest inhibition of alfalfa seedling establishment on soils continuously cropped to alfalfa is due to phytotoxins derived mainly from residues of alfalfa incorporated into the soil. Adsorbed substances on the soil colloids apparently have a minor contribution to the phytotoxicity in the soil. Extracts of crop residues caused a greater inhibition of seedling development than either whole or screened soils and were found to be low in pH and dissolved salts. Although residues from soil from old alfalfa stands were more inhibitory to seedling dry weight than residues from a soil cropped to alfalfa for one year, we suggest a management practice that allows regrowth of alfalfa shoots, regardless of the age of the stand, prior to plowing under would possibly introduce a large supply of phytotoxic material. This study supports evidence that red clover growth is stimulated and alfalfa growth is depressed on soils previously cropped to alfalfa (Jensen, 1984).

When extracts of alfalfa residue were examined by a fluorescence technique on developed TLC plates, the greatest inhibition to alfalfa root length was located in the region with $R_f = 0.78$. The greatest inhibition of alfalfa root length was observed in this region isolated from alfalfa residue screened from soils cropped to alfalfa for 10 years. Phytotoxic compounds, suspected to be phenolic in nature, could not be identified by gas chromatography of the crude alfalfa residue extract or the eluted band.

CONCLUSIONS

Our results do not indicate a specificity of response of alfalfa to phytotoxins extracted from alfalfa residues. However, we found that the phytotoxicity reportedly occurring from the decomposition of alfalfa residues in soil (McElgunn and Heinrichs, 1970; Jensen, 1984) is due to water-soluble substances. Bioassays utilizing TLC demonstrated decomposing alfalfa residues release phytotoxins that are not retained by soil colloids and that a high degree of autotoxicity is associated with residues collected from a soil cropped to alfalfa for 10 years.

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SKIN SURFACE MICROFLORA OF THE SADDLE-BACK TAMARIN MONKEY, *Saguinus fuscicollis*

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Abstract—The resident skin surface microflora of 12 male and three female *Saguinus fuscicollis* was studied. The suprapubic–circumgenital gland surface and the perirectal area were most heavily colonized (10^6 – 10^8 colony forming units/cm²), but high numbers of organisms were also present on the abdomen, the sternal gland surface, and palms and plantar surfaces. Bacteria were also recovered from hair clippings as well as from shaven skin surface, although at much lower densities (10^2 – 10^4 colony forming units/cm²). Coagulase negative staphylococci, gram-negative bacteria, *Streptococcus* species and coryneform bacteria were most dominant. *Staphylococcus aureus* and species of *Bacillus* were also present. Fungi, yeast, and dermatophytes were infrequently recovered or completely absent. The microbial flora of these tamarins appears to be closely associated with the secretions of the circumgenital scent gland and would therefore be ideally situated to participate in the generation or modification of chemical signals.

Key Words—Saddle-back tamarin, *Saguinus fuscicollis*, primates, Callitrichidae, surface microflora, chemical communication.

INTRODUCTION

Chemical communication, probably the oldest mode of communication among organisms, fulfills important behavioral and physiological functions (Vandenbergh, 1983). The possible role of microorganisms in the production of chemical signals has received increasing attention (Albone, 1984). Both aerobic and anaerobic microflora have been implicated in the production or modification of chemical signals. Gosden and Ware (1976) demonstrated that the aerobic bacterial flora of the anal sac of the red fox was composed of a wide range of organisms, including *Streptococcus* spp., *Proteus* spp., coliform bacteria, *Staphylococcus* spp., *Pseudomonas* spp., *Neisseria* spp. and *Bacillus* spp. The microflora of the inguinal pouches of the wild rabbit, *Oryctolagus cuniculus* is dominated by *Staphylococcus aureus* and the yeast *Candida kruzei*. Other isolates include *Bacillus subtilis*, *Escherichia coli*, and *Streptococcus faecalis* (Merritt et al., 1982). Albone et al. (1974) have compared the chemistry and the microbiology of the anal sac secretion of the red fox (*Vulpes vulpes*) with the anal sac secretion of the lion (*Panthera leo*) and demonstrated gross similarities in volatile and microbiological composition. Ware and Gosden (1980) have demonstrated that a variety of anaerobes, including species of *Bacteroides*, *Clostridium*, *Fusobacterium*, *Eubacterium*, *Peptostreptococcus*, and *Bifidobacterium* can also be isolated from the anal sac of the red fox.

Scent glands and scent-marking behavior are well developed in several groups of primates, among them the South American callitrichid monkeys (Epple et al., 1986). The saddle-back tamarin, *Saguinus fuscicollis*, a member of the family Callitrichidae, possesses an apocrine scent gland on the midchest, above the sternum, and a large, almost hairless pad of holocrine and apocrine glands in the circumgenital-suprapubic area (Perkins, 1966; Zeller et al., 1988). Males and females frequently scent mark with the suprapubic-circumgenital gland, depositing glandular secretions, urine, and possibly genital discharge (Epple et al., 1986). Behavioral studies have shown that scent marks communicate a variety of information relating to species, subspecies, gender, individual, endocrine condition, and social status (Epple et al., 1986).

It is conceivable that bacteria present on the glandular surface of the saddle-back tamarin are involved in the production or modification of the volatile constituents of the scent marks. The present study represents a first step in elucidation of this possibility: the characterization of the microbial flora.

METHODS AND MATERIALS

The first phase of this study involved three female and three male tamarins (group 1). The animals were sedated with 0.2 ml ketamine hydrochloride, 100 mg/ml (Bristol Laboratories, Bristol-Meyers, Syracuse, New York) and sam-

pled for the presence of aerobic and anaerobic microflora. Gloves were worn when handling and sampling the animals. Samples were taken from the suprapubic gland surface (scent pad), the sternal gland surface, the perirectal area, palm and planar surface, back (unshaven), and abdomen (unshaven).

Cyclical changes in the skin gland secretions may occur in females, which may in turn have an effect on the bacterial population. Therefore in the second phase of the study an additional nine males only (group 2) were studied in the following year for the presence of bacteria on the scent pad, the sternal gland, and the perirectal region. Samples from the back were also obtained from three of these animals after the hair had been cut from an area of 3 cm². The hairs were also collected for microbiological examination.

For sampling of the skin surface, a sterile cotton-tipped swab was moistened with 2 ml of sterile 0.1% Tween 80 and was rotated five times in a clockwise direction and then five times counterclockwise on an area of approximately 3 cm². The swab was returned to the culture tube, and the procedure was repeated four times. For the isolation of anaerobic bacteria, the swabs were placed into 2 ml, sterile, prereduced thioglycollate broth (BBL, Becton Dickinson, Cockeysville, Maryland) with 0.1% Tween 80. The hair clippings were placed directly into the 2 ml of sampling fluid.

In the laboratory, 10-fold dilutions of the samples were made in 0.05% Tween 80 or thioglycollate broth and appropriate dilutions were drop-plated onto the following media: (1) Trypticase soy agar with 5% sheep blood and 0.5% yeast extract—general nutrient medium for aerobes and anaerobes; (2) blood agar with 0.5% Tween 80—general nutrient medium; (3) MacConkey agar—selective isolation of Gram-negative rods; (4) phenylethylalcohol agar with 5% sheep blood—selective isolation of Gram-positive organisms; (5) manitol salt agar—selective isolation of *Staphylococcus aureus*; (6) Mycosel agar—selective isolation of dermatophytes; (7) Sabouraud dextrose agar with 0.05% chloramphenicol— isolation of fungi; (8) Schaedler agar with 0.1% Tween 80 and standard salt solution—anaerobic medium; and (9) Rogosa SL agar—selective isolation of lactobacilli. All media were from BBL. Aerobic plates were incubated at 35°C for three days, and then at room temperature for another four days. Anaerobic plates were incubated for seven days at 35°C in anaerobic jars in an anaerobic atmosphere enriched with CO₂ (BBL GasPak, Anaerobic Systems). All media for isolation of anaerobes were prereduced prior to inoculation by incubation in an anaerobic atmosphere at 4°C.

Organisms were identified according to colony morphology, Gram stain reaction, and biochemical tests, which were performed on washed 3-day-old bacteria inoculated as 25 μl of a turbid suspension of 0.15 M NaCl according to standard identification procedures (Paik, 1980). Tests for nitrate reduction and hydrolysis of indole and urea were performed in Stuart's medium. Catalase production was tested by the slide method, on bacteria grown on Trypticase soy agar with 0.5% yeast extract. All other tests were performed using the API

identification system: API 20E enteric system, API Staph indent, and API 20S *Streptococcus* system (Analytab Products, Plainview, New York). Non-spore-forming, catalase-positive, aerobic, Gram-positive rods were identified as coryneform bacteria. Yeast were identified to the genus level, and fungi were identified on the basis of macromorphology.

Three 4-mm punch biopsies were taken from the suprapubic gland of one male *S. fuscicollis* that had been sacrificed. Thin sections were processed and examined for the presence of bacteria using scanning electron microscopy according to Lehmann et al. (1983).

RESULTS

High numbers of bacteria were present on all body sites of male and female saddle-back tamarins (Table 1). The composition and the density of the flora of the three males (group 1) that were sampled for comparison with the three females in the first phase of the study were similar to the second group of nine males (group 2) sampled at a later date (Tables 2 and 3).

The flora was dominated by aerobic and facultatively anaerobic bacteria; anaerobic isolation did not yield any strict anaerobes. Electron microscopy of thin sections of the suprapubic gland of the sacrificed animal showed the presence of large multiacinar sebaceous glands devoid of any bacteria in the glands or at the opening of the glandular ducts.

Coagulase-negative staphylococci, Gram-negative bacteria, *Streptococcus* species, and coryneform bacteria were the predominant organisms in both males and females (Tables 2 and 3). *S. aureus* was present in lower numbers, and

TABLE 1. DENSITY OF MICROFLORA ON BODY SITES OF *Saguinus fuscicollis* EXPRESSED AS Log_{10} COLONY FORMING UNITS/ cm^2

Site	Females (<i>N</i> = 3)	Males	
		Group 1 (<i>N</i> = 3)	Group 2 (<i>N</i> = 9)
Suprapubic	6.1 ± 0.8	6.9 ± 0.3	6.5 ± 0.8
Perirectal	7.8 ± 1.0	7.3 ± 0.5	6.3 ± 0.6
Abdomen	6.4 ± 0.8	6.5 ± 0.4	
Sternal	4.8 ± 0.6	5.5 ± 0.2	4.7 ± 0.5
Back	4.9 ± 0.6	5.3 ± 0.3	— ^a
Palm	5.7 ± 0.8	6.1 ± 0.5	
Plantar	6.1 ± 1.0	6.2 ± 0.4	

^aMinus (—) indicates that organisms were not present.

TABLE 2. DENSITY OF MICROORGANISMS ON BODY SITES OF 3 MALE AND 3 FEMALE *S. fuscicollis* EXPRESSED AS \log_{10} CFU/cm²

Site	SA ^a	CNS	GNB	CF	F/Y	STR	ASF
Suprapubic	2.4 ± 0.7 ^b	4.7 ± 1.1 5.1 ± 0.9	4.1 ± 0.9 3.0 ± 1.0	4.1 ± 1.4 4.2 ± 1.6	— —	3.7 ± 0.5 2.8 ± 0.6	— —
Abdomen	2.3 ± 0.2 1.9 ± 0.5	4.5 ± 0.4 4.4 ± 1.3	4.3 ± 0.5 4.3 ± 0.2	4.1 ^c 5.1 ± 1.1	— —	3.2 ± 1.7 4.3 ± 0.4	1.4 ^c —
Perirectal	— 1.1 ^d	3.6 ± 1.7 5.5 ± 0.5	4.7 ± 0.8 6.1 ± 0.8	5.9 ± 0.3 5.0 ± 1.6	1.1Y ^c —	5.6 ± 1.0 6.0 ± 0.2	— —
Back	1.8 ± 0.3 —	3.4 ± 0.6 3.1 ± 0.4	3.8 ± 0.6 3.1 ± 0.7	3.0 ^d 3.4 ± 0.2	— —	3.7 ^c 3.0 ± 0.2	— —
Sternal	1.5 ^c 1.1 ^d	3.4 ± 0.3 2.7 ± 0.04	3.6 ± 0.6 3.4 ± 0.3	3.5 ^d 3.4 ± 0.2	— —	3.1 ± 0.6 3.1 ± 0.6	— —
Palm	1.9 ^c 2.0 ^d	3.6 ± 1.5 3.0 ± 0.1	4.4 ± 0.7 3.7 ± 0.6	3.9 ^c 5.1 ± 0.4	— Females	3.1 ^c 4.1 ± 1.1	— 1.4 ^d
Plantar	2.6 ^d 2.7 ± 1.6	4.0 ± 1.1 3.7 ± 0.3	3.6 ± 0.7 3.6 ± 1.5	3.9 ^c 5.2 ± 0.9	— Females	3.8 ± 0.1 4.5 ± 1.4	— —

^aSA = *Staphylococcus aureus*, CNS = coagulase-negative staphylococci, GNB = gram-negative bacteria, CF = coryneform bacteria, F = fungi, Y = yeast, Str = streptococci, ASF = aerobic spore formers.

^bTop figures = males; bottom figures = females; minus (—) indicates that organisms were not present.

^cN = 2.

^dN = 1.

TABLE 3. FREQUENCY OF ISOLATION AND DENSITY OF MICROORGANISMS FROM MALE *S. fuscicollis* (N = 9, Group 2)

Site	SA ^a	CNS	GNB	CF	F/Y	STR	ASF
Frequency of isolation (%)							
Suprapubic	33	89	89	56	44	100	56
Sternal	33	100	100	44	11	100	56
Perirectal	22	78	100	33	78	89	22
Skin (shaven) ^b	33	100	100	— ^c	—	100	67
Hair ^b	33	100	100	—	—	100	67
Density (log ₁₀ CFU/cm ²)							
Suprapubic	3.6 ± 0.5	4.9 ± 1.0	4.9 ± 0.9	5.3 ± 1.3	1.9 ± 0.2Y	6.2 ± 0.2	2.9 ± 0.9
Sternal	2.2 ± 0.6	3.1 ± 1.0	2.0 ± 0.5	2.9 ± 0.9	2.4Y ^d	4.6 ± 0.5	2.6 ± 0.6
Perirectal	3.4 ± 1.3	4.1 ± 1.1	5.0 ± 0.9	4.9 ± 0.9	3.2 ± 1.1Y	5.9 ± 0.8	3.2 ± 1.1
Skin (shaven) ^b	1.4 ^d	1.9 ± 0.6	2.1 ± 0.5	—	—	2.9 ± 0.9	1.4 ^d
Hair ^b	2.4 ^d	2.1 ± 0.2	2.8 ± 0.6	—	—	4.2 ± 0.7	0.3 ^e

^aSA = *Staphylococcus aureus*, CNS = coagulase-negative staphylococci, GNB = gram-negative bacteria, CF = coryneform bacteria, F = fungi, Y = yeast, STR = streptococci, ASF = aerobic spore formers.

^bN = 3.

^cMinus (—) not present.

^dN = 1.

^eN = 2.

yeast, fungi, and anaerobic spore formers were occasionally recovered. No dermatophytes were present. The overall composition of the flora of males and females was very similar. Seven species of coagulase-negative staphylococci were tentatively identified: *S. haemolyticus* (with an overall prevalence of 72%), *S. epidermidis* (61%), *S. simulans* (56%), *S. warnerii* (37%), *S. saprophyticus* (26%), *S. sciuri* (2%), and *S. intermedius* (2%). Of the Gram-negative bacteria, *Pseudomonas fluorescens* was most frequently isolated at a 96% prevalence, followed by *Escherichia coli* (72%), *Proteus mirabilis* (35%), *Acinetobacter calcoaceticus* (33%), and *Serratia rubidea* (7%). Two streptococcal species were isolated: *S. faecium* and *S. faecalis* with a prevalence of 93% and 89%, respectively. Coryneform bacteria were also commonly isolated (63%). Members of the genus *Bacillus* (27%) represented the aerobic spore formers, but these were not identified to the species level. Similarly, species of the yeast *Candida* were recovered at a 13% isolation frequency. The fungi were identified as members of the genus *Penicillium* and were present in three of the 75 samples.

The most prevalent organisms on the surface of the shaven skin and on the hair clippings were the coagulase-negative staphylococci, Gram-negative bacteria, and the streptococci (Table 3). The densities of these organisms on the skin and the hair were also very similar, but the streptococci were recovered in highest densities from hair clippings.

DISCUSSION

The resident microflora of the saddle-back tamarin is dominated by coagulase-negative staphylococci, Gram-negative bacteria, streptococci, and coryneform bacteria. It is possible that this flora is, at least in part, of fecal origin (Lewis et al., 1987). The isolation rates of *S. aureus* and *Bacillus* species were more variable. This suggests that these organisms are part of a transient flora and may be transferred from the personnel caring for the tamarins or from the surrounding environment. The fungi also appear to be part of environmental contamination. The humid and warm environments of the scent pad, perirectal area, and the abdomen stimulate high bacterial proliferation. Thus, the bacteria are ideally situated for interacting with secretions of the suprapubic-circumgenital gland. The examination of the samples taken from shaven skin and hair clippings showed that the numbers of bacteria were higher on the hair. In contrast, Leyden et al. (1981) have demonstrated that in the human axilla, insignificant numbers of bacteria are present on the hairs and that these are translocated from the abundant resident population on the skin. Since hairs are a poor substrate for growth of bacteria, it is likely that in the tamarins the majority of organisms on sites other than the glandular areas, abdomen, and

perirectal area are translocated by contact with other members of the group and with the environment, rather than originating from the surface of the skin.

Yarger et al. (1977) and Belcher et al. (1986) have documented the presence of butyrate esters (C_{16} – C_{24}) and squalene in scent marks of *Saguinus fuscicollis*. More recently, the presence of some short-chain fatty acids, steroid hormone metabolites, and proteins has been established (Belcher et al., unpublished results). Albone et al. (1974) have attributed the presence of various short-chain fatty acids in anal sac secretions to microbial action on carbohydrates and proteins. Short-chain fatty acids, although present, are not among the major volatile components of *S. fuscicollis* scent marks. However, bacteria that reside on human skin (Kearney et al., 1984) actively hydrolyze triglycerides to free fatty acids, which fall in the molecular weight range of those acids that are detected in the scent marks from *S. fuscicollis*. Bacterial cell wall fatty acids typically include these chain lengths (Goodfellow and Minnikin, 1985), hence, it is possible that bacterial debris contributes to the composition of the scent marks. Bacteria are also capable of degrading cholesterol, and it has been suggested that bacteria also esterify cholesterol into cholesteryl esters (Turtiff, 1948; Puhvel, 1975). Nixon et al. (1984) have reported that bacteria that colonize the human axilla are capable of metabolizing testosterone with the subsequent production of 17β -hydroxy- 5α -androstan-3-one and 17β -hydroxy- 5β -androstan-3-one, and according to Labows et al. (1982), several authors have demonstrated that the androgen steroids account for some of the odors of the human axilla. It is possible that bacteria similarly metabolize androgens in the deposited scent marks of the marmoset.

Tamarins in this study lived in social groups and were not separated from one another prior to the study. Translocation of the bacterial flora by contact probably occurs also in nature and thus is part of the behavior of these primates. Albone and Perry (1975) have emphasized the importance of translocation of the microbial flora within the population, and speculated that a group of animals living together would be expected to share a common microflora that characterizes the group. If the microorganisms are responsible for the production of specific volatiles, these may serve as important signals that are characteristic to the group and the microflora that it shares.

The bacterial flora of the saddle-back tamarins includes several species that have also been isolated from other mammals, such as the red fox (Albone et al., 1974; Gosden and Ware, 1976) and the rabbit (Merritt et al., 1982), in which chemical communication is important. Streptococci, namely *Strep. faecium* and *Strep. faecalis*; staphylococci, *S. aureus* and the coagulase-negative staphylococci, Gram-negative bacteria; *E. coli*; and *Proteus mirabilis* have all been reported to be associated with the glandular regions responsible for the generation of the signals. Previous authors have not attempted to speciate the coagulase-negative staphylococci that have been isolated. We found, using the

API identification system, that the organisms could be tentatively assigned to the following seven species: *S. haemolyticus*, *S. epidermidis*, *S. simulans*, *S. warnerii*, *S. saprophyticus*, *S. sciuri*, and *S. intermedius*. Although speciation of these organisms is tentative at this point, it should be pointed out that Brun et al. (1978) have reported excellent correlation between the identification method of Kloos and Schleifer (1975) and the API system.

Several authors have reported that anaerobes form a resident bacterial flora found in sacs, pouches, and invaginations of many other species that are known to depend on chemical communication (Albone, 1984; Svendsen and Jollick, 1978; Ware and Gosden, 1980). The present study indicates that the saddle-back tamarin is heavily colonized by aerobic and facultatively anaerobic bacteria. However, the suprapubic scent gland consists of a pad of glandular skin in which pouches and invaginations are not present (Epple et al., 1986). The gland surface is constantly exposed to air. It thus would be highly unlikely that strict anaerobes, such as *Bacteroides* spp. or *Fusobacterium* spp. (Ware and Gosden, 1980; Merritt et al., 1982) found in other species, would be present on *Saguinus fuscicollis*. Electron microscopy of thin sections from the scent pad confirmed that no organisms were present in the glands or glandular ducts, where an anaerobic environment might prevail. To confirm these observations, further work will be conducted involving sampling the tamarins in an anaerobic environment, as described by Ware and Gosden (1980). This will be the subject of a future communication.

In summary the skin surface microflora of *Saguinus fuscicollis* is composed of aerobic and facultatively anaerobic bacteria. High numbers of coagulase-negative staphylococci, Gram-negative bacteria, streptococci and coryneform bacteria are consistently recovered. The suprapubic scent gland and the perirectal area are most heavily colonized; thus the bacteria are ideally situated to participate in the generation or modification of chemical signals. In vitro studies on the production of volatiles by bacteria in the presence of scent-gland secretions are currently being pursued.

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AGE-SPECIFIC TITER AND ANTENNAL PERCEPTION
OF ACETIC ACID, A COMPONENT OF MALE
Pseudaletia unipuncta (HAW.)¹
HAIRPENCIL SECRETION

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Abstract—Hairpencil secretion of *Pseudaletia unipuncta* (Haw.) contains acetic acid as well as previously identified benzaldehyde and benzyl alcohol. Age-specific titers of acetic acid were significantly greater than those of benzaldehyde and, at 25°C, accumulation of both compounds in the hairpencils peaked on the second day after emergence. Excised antennae of males and females perceived both compounds. Antennal response to acetic acid did not vary significantly with age or sex, but male response to benzaldehyde was significantly greater than female response at all ages tested. Antennal response of both males and females to benzaldehyde tended to be greater on the fourth and eighth days after emergence than on the second.

Key Words—*Pseudaletia unipuncta*, Lepidoptera, Noctuidae, hairpencils, male pheromone, acetic acid, benzaldehyde, benzyl alcohol, antennal response.

INTRODUCTION

Studies of pheromone composition and perception in Lepidoptera usually focus on female pheromones which, due to their role as male attractants, have considerable potential in pest management programs. Male pheromones, on the other hand, have little evident applicability and have therefore received less

¹Lepidoptera: Noctuidae.

attention. The deployment and function of male pheromones varies among species, although most are released just prior to copulation and promote successful courtship (Fitzpatrick and McNeil, 1988).

Pseudaletia unipuncta male pheromone is released from abdominal hair-pencils as the male attempts genital contact with the female (Fitzpatrick and McNeil, 1988). The pheromone facilitates copulation (Farine, 1982), apparently by inhibiting female rejection behavior (Fitzpatrick and McNeil, 1988). It has also been suggested that *P. unipuncta* male pheromone inhibits conspecific males from approaching and attempting to copulate with calling females (Hirai et al., 1978). However, results of more recent work fail to support this hypothesis (Fitzpatrick et al., 1988).

It has been known for some time that *P. unipuncta* male pheromone contains benzaldehyde and benzyl alcohol (Grant et al., 1972) and that antennae of both sexes respond to these compounds (Grant et al., 1972; Seabrook et al., 1979). Benzaldehyde was believed to be the only active pheromone component; it is enzymatically derived from benzyl alcohol during the two days following emergence (Clearwater, 1975; Hirai, 1980) and is thereafter reported to be the major component of hairpencil secretion (Grant et al., 1972; Hirai, 1980; Fitzpatrick et al., 1985). However, Grant et al. (1972) reported a third, unidentified compound that was resolvable only on a polar column and had a retention time less than that of benzaldehyde.

During routine analyses of *P. unipuncta* male pheromone, we also noticed an additional peak, which proved to be acetic acid, the major component of male scent. Here we report its titer as a function of male age and its perception by antennae of both sexes.

METHODS AND MATERIALS

Insects. *P. unipuncta* males came from a laboratory population established from mated females collected at Normandin, Québec, Canada. Larvae were individually reared on a pinto bean diet (modified from Shorey and Hale, 1965), sexed after pupation, and the sexes maintained in separate incubators thereafter. Adults were kept individually in 16-dram clear plastic vials where they had continual access to 8% sucrose. All stages were maintained at $25 \pm 1^\circ\text{C}$, $65 \pm 5\%$ relative humidity, under a 16:8 light-dark photoperiod.

Identification of Acetic Acid. Four-day-old males were inactivated by chilling at -10°C for 15 min, a procedure that does not affect hairpencil content (Fitzpatrick et al., 1985). Hairpencils were quickly removed with fine forceps and placed in chilled Teflon-capped 1-dram amber vials containing methylene chloride (two hairpencils per male per vial). All hairpencil excision took place

during the last hour of scotophase, which corresponds to the end of the period of sexual activity at this temperature and photoperiod.

Identification of acetic acid was carried out by capillary gas-liquid chromatography (GLC) and verified by gas chromatography-mass spectroscopy (GC-MS). GLC analyses were conducted on a Hewlett Packard 5890 GLC fitted with flame ionization detectors and coupled to a Hewlett Packard 3392A integrator. The acetic acid peak, which first became apparent during low-temperature runs on a nonpolar methyl silicone column, was best resolved on a polar DX-4 column (0.25 mm \times 15 m) using hydrogen as the carrier gas. Injections to the DX-4 were made in split mode, and temperature was increased from 60°C to 125°C at 40°C/min after an initial hold of 1 min. GC-MS verification was conducted on a Kratos MS-80 using nonpolar DB-1 and polar DX-3 columns, with helium as the carrier gas. Mass spectra of insect-derived compounds were compared to those of standards, and the identity of acetic acid confirmed.

GLC Quantification of Acetic Acid as a Function of Male Age. Hairpencils of newly emerged (day 0) and 1-, 2-, 4-, 6-, 8-, and 10-day-old males were excised and placed in solvent-containing vials as described above. A known quantity of the internal standard, naphthalene, was added to each vial, and the vials stored at -10°C for up to nine days before analysis. Acetic acid was quantified by comparing electronically integrated acetic acid peak areas with those of naphthalene. Two injections from each sample were analyzed, and results averaged to yield amounts of acetic acid per male. Benzaldehyde titers were analyzed in the same manner.

Electroantennograms. EAGs were recorded on an apparatus similar to that described by Nagai et al. (1977). Odor stimuli were delivered from glass cartridges (5 mm \times 5 cm) in 2-sec puffs along the longitudinal axis of the antennae. Just prior to use, cartridges were loaded with 10 μ l of the test dilution and rolled, to ensure even distribution of compounds, until all liquid had evaporated. Each cartridge was used only once.

Excised antennae from 2-, 4- and 8-day-old males and females were tested for their response to each compound during the last 3 hr of scotophase. By testing individuals of these ages, we were able to monitor antennal perception from the onset of sexual activity to mid-life, since male response to female pheromone (Turgeon et al., 1983), male reproductive activity (Fitzpatrick and McNeil, 1989) and female calling behavior (Turgeon and McNeil, 1982) do not begin before the second day after emergence at 25°C. Acetic acid, dissolved in methylene chloride, was applied to cartridges at a concentration of 2.5×10^2 μ g/10 μ l. Preliminary tests showed that antennal responses of both sexes to concentrations less than this did not differ from responses to control cartridges loaded with methylene chloride and that 5×10^2 μ g/10 μ l exceeded saturation. Benzaldehyde, 96% pure by GLC, was applied to cartridges at a

concentration of 10^2 $\mu\text{g}/10$ μl of methylene chloride. This did not exceed saturation, but was as concentrated as possible because, at higher concentrations, benzoic acid accumulated in cartridges and antennal response of both sexes decreased. Each antenna was subjected to three consecutive stimuli: (1) airflow from an empty cartridge (to ensure that the response of mechanoreceptors stimulated by the airflow could be recorded); (2) methylene chloride control, and (3) acetic acid or benzaldehyde dilution. There was a 3-min recovery period between stimuli. The EAG response was calculated as the response to acetic acid or benzaldehyde minus the response to methylene chloride.

RESULTS

Analysis of hairpencil content as a function of male age showed that acetic acid titer tracked that of benzaldehyde, rising to a peak two days after emergence, decreasing from day 4 to day 6, then subsequently increasing (Figure 1). Acetic acid titer was significantly higher than benzaldehyde titer at all ages (two-way ANOVA; $F = 27.38$, $P < 0.001$, $df = 1, 128$). This difference may even be underestimated, as subsequent work has shown that, although neither benzaldehyde or benzyl alcohol evaporate during storage at -10°C , some acetic acid may be lost. Benzyl alcohol, the precursor of benzaldehyde, peaked on day 1 and was not detectable after day 6 (Figure 1).

Electroantennogram recordings indicated that both male and female antennae respond to acetic acid (Figure 2A). Female response decreased gradually

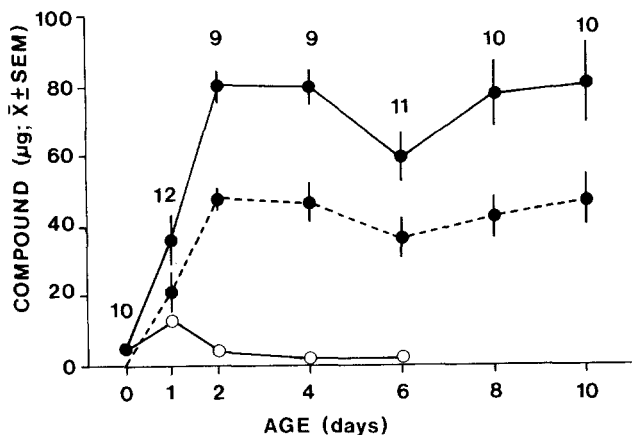


FIG. 1. Titers of acetic acid (—●—), benzaldehyde (---●---), and benzyl alcohol (—○—) in diet-fed, virgin *P. unipuncta* males. The number of males analyzed per age is given on the upper graph.

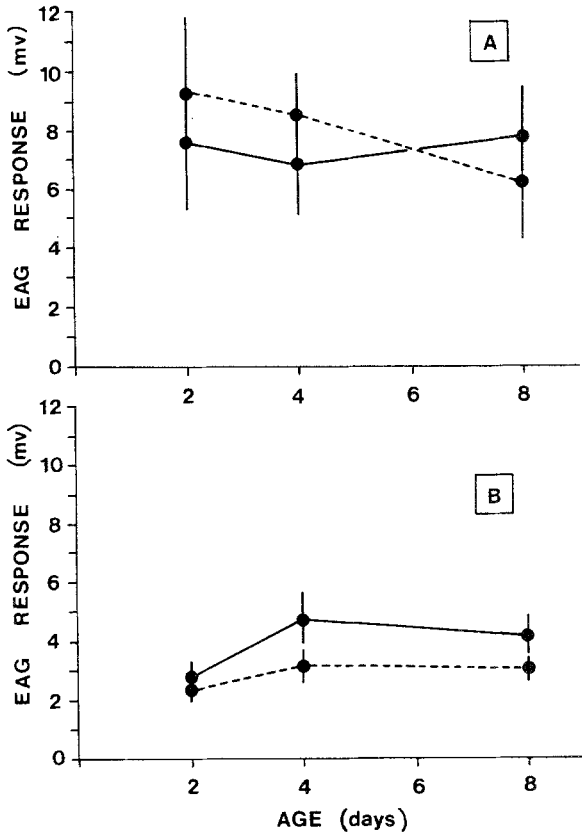


FIG. 2. EAG responses of excised male (—●—) and female (---●---) *P. unipuncta* antennae to source concentrations of (A) $2.5 \times 10^2 \mu\text{g}$ of acetic acid and (B) $10^2 \mu\text{g}$ of benzaldehyde dissolved in $10 \mu\text{l}$ of methylene chloride. Six individuals were tested per age, sex, and compound.

with age, although the decline was not significant (ANOVA; $F = 0.67$, $P = 0.53$, $df = 2, 15$), while male response remained relatively constant (ANOVA; $F = 0.07$, $P = 0.93$, $df = 2, 16$). There were no differences between male and female EAG responses (two-way ANOVA; $F = 0.14$, $P = 0.71$, $df = 1, 31$). Antennae of both sexes also responded to benzaldehyde (Figure 2B), as reported by Grant et al. (1972) and Seabrook et al. (1979). Both male and female responses were greater on days 4 and 8 than on day 2, although these increases were not significant for either sex (ANOVA; males: $F = 2.12$, $P = 0.16$, $df = 2, 17$; females: $F = 1.02$, $P = 0.38$, $df = 2, 17$). However, male responses

to benzaldehyde were significantly higher than female responses (two-way ANOVA; $F = 5.04$, $P = 0.03$, $df = 1, 30$).

Waveforms to both compounds approximated normal EAGs (Boeckh et al., 1965): rapid depolarization followed by slower repolarization (recovery phase). Responses to acetic acid were sometimes characterized by a period of hyperpolarization after repolarization.

DISCUSSION

Acetic acid has not been reported in the male scent of any other lepidopterans, although similar low-molecular-weight carboxylic acids have been identified in scent brushes of several noctuids and one geometrid (Aplin and Birch, 1970). *Leucania impura* (Hueb.), *L. conigera* (Schiff.), and *L. pallens* (L.), all in the Hadeninae, the same subfamily as *P. unipuncta*, produce isobutyric acid. In these three species, benzaldehyde is the major scent brush component and isobutyric acid makes up 20% of the secretion (Aplin and Birch, 1970).

In *P. unipuncta* males, acetic acid predominates, both in terms of mass (Figure 1) and of number of molecules. Given that the molecular weight of acetic acid is 60 and that of benzaldehyde 106, each microgram of acetic acid contains 1.77 times as many molecules as each microgram of benzaldehyde. Thus on day 4, when scent brushes contain on average 80 μg of acetic acid and 47 μg of benzaldehyde, the molecular ratio is approximately 3 : 1. When scent is released just before a male's first copulation, the molecular ratio of the released compounds is lower but still favors acetic acid. On average, 6 μg of acetic acid and 8 μg of benzaldehyde are given off (Fitzpatrick and McNeil, 1988) i.e., 1.34 molecules of acetic acid per molecule of benzaldehyde.

The temporal pattern of acetic acid accumulation during the two days after emergence follows that of benzaldehyde (Figure 1). During this period, benzaldehyde is being enzymatically converted from benzyl alcohol (Clearwater, 1975; Hirai, 1980; Miller et al., unpublished results), which is in turn a degradation product of a glycoside precursor (Clearwater, 1975) emptied onto the hairpencils from the Stobbe glands at emergence (Birch, 1970; Clearwater, 1975; Miller et al., unpublished results). Storing benzaldehyde as a glycoside circumvents its toxic effects on cell metabolism (Clearwater, 1975). It is therefore probable that acetic acid is also stored as a nontoxic precursor until enzymatic conversion. Research on this possibility is presently underway.

Antennae of both males and females perceive acetic acid. EAG responses do not vary significantly with age or sex, although the response of female antennae tends to decline slightly with age. Benzaldehyde is also perceived by antennae of both sexes, and our data show that male response to benzaldehyde is significantly greater than the response of females, contrary to the results of

Grant et al. (1972), but in agreement with those of Seabrook et al. (1979). Given that the EAG is thought to be the summed potential of activated receptor cells of the antenna (Boeckh, 1969), our results suggest that male antennae possess more acceptor sites or receptor cells for benzaldehyde than do female antennae. Seabrook et al. (1979) also reported that peak sensitivity to benzaldehyde occurs at a much younger age in males than in females (day 3 vs. day 8–9).

Both benzaldehyde and acetic acid are structurally similar to compounds that may serve as feeding stimuli. Many noctuids are attracted to exudates of tree wounds (R. Webster, personal communication), which may contain phenols (Kevan et al., 1983) and, depending on the type of bacterial fermentation present (Lehninger, 1975), low-molecular-weight carboxylic acids. *P. unipuncta* is one of a number of noctuids attracted to phenylacetaldehyde, a phenolic very similar to benzaldehyde, given off by the bladder flower (Cantelo and Jacobson, 1979). There is as yet no general agreement on the type(s) of receptor cells used to perceive male pheromone components (e.g., Birch, 1971; Grant, 1971; Priesner, 1979; Seabrook et al., 1979). However, if male scent must be distinguished from closely related food odors, the responsiveness of the receptor cells involved may vary as a function of factors such as the physiological state (e.g., Davis, 1986) and sex of the perceiver, as well as time of day and relative proportions of the two compounds. This possibility awaits further electrophysiological and behavioral studies.

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URINARY VOLATILE PROFILES OF PINE VOLE, *Microtus pinetorum*, AND THEIR ENDOCRINE DEPENDENCY

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Abstract—The volatile compounds identified by combined gas chromatography-mass spectrometry in *Microtus pinetorum* urine include alcohols, aldehydes, hydrocarbons, ketones, nitriles, and pyrazines. Several lactone derivatives were found to be characteristic urinary substances of this species. Ovariectomy depressed concentrations of only five out of a great number of profile constituents. Elevating estrogen levels (by exposing females to male-soiled bedding or treating them with estradiol) tends to depress the urinary concentration of a number of selected volatiles. Estrogen implantation provoked a periodic increase in the level of three compounds (nonanal, benzaldehyde, and an unidentified substance). The volatile profile of castrate male urine was similar to that of intact male urine. Female urine contained γ -octanoic lactone and two pyrazine derivatives in higher concentrations and *p*-methyl-propenylbenzene in a lower concentration, when compared to male urine. No qualitative differences between the urinary profiles of males and females were observed.

Key Words—Pine voles, *Microtus pinetorum*, capillary gas chromatography, urinary chemosignals.

INTRODUCTION

It is becoming increasingly clear that both small and large molecules can act as mammalian pheromones. Some attempts to isolate the active fraction of urine and to structurally characterize these chemosignals have been successful in murines, with a particular focus on the house mouse, *Mus musculus*. The male's

ability to hasten the onset of puberty in female mice is mediated by a high-molecular-weight, nonvolatile urinary fraction (Vandenbergh et al., 1975, 1976). The male-produced chemosignals involved in the estrous synchronization effect appear to be volatile (Jemiolo et al., 1986). The female mouse urinary cue that inhibits puberty onset in other females (Drickamer, 1982a,b, 1983) is a mixture of urinary volatile compounds (Novotny et al., 1986).

Similar to spontaneously ovulating murines, the inductively ovulating microtines are also sensitive to reproductive activation by urinary chemosignals from conspecifics. Actual contact of a juvenile and an adult female vole with a strange mature male or his urine provides a particularly potent stimulus for her reproductive development (Clarke and Clulow, 1973; Richmond and Stehn, 1976; Carter et al., 1980; Baddaloo and Clulow, 1981; Milligan, 1982; Jemiolo, 1987). Suppression of puberty in voles can occur in animals reared in mixed-sex or same-sex groups (Batzli et al., 1977; Getz et al., 1983).

While it has also been demonstrated that urinary volatile constituents are involved in the control of reproductive activities of female mice, no similar chemical studies have been performed on microtines. The present study has been undertaken to investigate the urinary profiles of volatile substances excreted by female and male pine voles, *M. pinetorum*, and their dependence on the endocrinological status of the investigated animals. Selected volatiles were measured quantitatively by established chromatographic methods and identifications were made through mass spectrometry followed by verification through chromatographic retention of authentic compounds.

METHODS AND MATERIALS

Animals. All pine voles, *Microtus pinetorum*, used in this study were from a laboratory-bred colony. The voles were housed individually in plastic cages (30 × 15 × 13 cm) under conditions of controlled temperature (21°C) and light (0600–2000 hr). They were fed Purina Rat Chow and Wayne Guinea Pig Diet ad libitum, supplemented daily by a slice of apple and carrot. Water was constantly available. Bedding was changed weekly.

The animals under investigation were a gift from Dr. John Vandenbergh of North Carolina State University, Raleigh, North Carolina.

Sample Collection. Urine samples used for the chemical analysis were obtained by placing voles into drip-metabolism cages. Each collection period, lasting 6 hr, was begun at the same time of the day, i.e., from 0900 to 1500 hr. Urine from each metabolism cage drained into a collection vessel, which was maintained on Dry Ice to ensure immediate freezing of the sample. Samples were stored at –20°C until analysis. All urine samples were collected between the months of May and September.

Sample Analysis. Urinary volatiles were concentrated onto a porous polymer, Tenax GC (Applied Science Laboratories, State College, Pennsylvania) using the headspace technique developed by Novotny et al. (1974). Purified helium gas at a flow rate of 100 ml/min sparged the volatiles from 1.0-ml urine samples for 1 hr. The headspace sampling was performed at room temperature. Volatiles were absorbed onto a precolumn packed with 4 mg of Tenax GC. Following sampling, volatiles were desorbed into the heated injection port (240°C) of a gas chromatograph (Series 1400, Varian Instruments, Walnut Creek, California) equipped with a flame ionization detector, and retrapped into a liquid-nitrogen-cooled section of a glass capillary column (60 m × 0.25 mm, ID), which had been statically coated with UCON-50-HB-2000 (Supelco, Inc., Bellefonte, Pennsylvania). Column temperature was programmed from 30°C to 160°C at a rate of 2°C/min. Relative quantitative comparisons of peak areas were made with a Perkin-Elmer Sigma 10 Gas Chromatography Data Station (Perkin-Elmer, Norwalk, Connecticut).

Volatile constituents were identified through capillary GC-MS (Hewlett-Packard 5981 dodecapole mass spectrometer), using electron impact ionization at 70 eV. In addition, the components were verified through retention-time measurements of authentic compounds.

Study 1: Volatile Profiles of Female Pine Vole Urine. All *M. pinetorum* females were between 90 and 120 days of age and were virgin at the beginning of the study. Urine was collected from four groups of animals: (1) unexposed females ($N = 7$), which were kept alone in their home cage for at least four weeks before urine collection; (2) exposed females ($N = 7$), which were placed for 24 hr in a cage previously used by unfamiliar, mature males for at least one week; (3) ovariectomized females ($N = 4$) that were gonadectomized under methoxyflurane anesthesia four weeks before urine collection; and (4) ovariectomized and estradiol-treated females; ovariectomy was performed four weeks before the estradiol capsule was implanted. A capsule containing 17 β -estradiol (Sigma Chemical Company, St. Louis, Missouri), was 5.0 mm long and prepared from Silastic Medical Grade Tubing (Dow Corning Corp., Midland, Michigan). The ends of the capsule were sealed with Silastic Medical Adhesive (Silicone Type A, Dow Corning) so as to leave a compartment 2–3 mm in length. This technique permits delivery of relatively precise and small quantities of hormone over a long period of time (Henderson et al., 1977; Moore, 1981). A capsule was implanted subdermally into the back of the neck of each female ($N = 6$). Urine collection was made at 48, 72, 96, 120, 144, and 312 hr after implantation. Urine collected from intact and ovariectomized females was used to establish a baseline. The collection from intact and ovariectomized females is represented at 01 and 02 hr after implantation.

Study 2: Volatile Profiles of Male Pine Vole Urine. All *M. pinetorum* males, sexually inexperienced at the beginning of this study, were 90–120 days of age.

Urine was collected from two groups of males: (1) intact ($N = 7$) and (2) castrated ($N = 5$), all housed separately. Males were castrated at least four weeks prior to the first urine collection.

Statistical Analysis. Statistical comparisons of the levels of excreted volatiles were made using t test and one-way analysis of variance (ANOVA). The number of samples run for the first three groups of females was 7, 7, and 4, respectively (1 ml of urine from each female). For the estrogen-treated females, a single run for a different time was evaluated (1 ml of urine was collected from six females for each period of time after implantation). The number of samples run for male urine was 7 for the intact and 5 for the castrated animals.

RESULTS

The capillary gas chromatograms shown in Figure 1 are representative of pine vole urinary volatile profiles obtained from (A) unexposed females, (B) females exposed to male-soiled bedding, and (C) intact males. Twenty-three volatile compounds were positively identified by combined capillary gas chromatography-mass spectrometry and retention measurements of authentic compounds (Table 1). Identified substances include pyrazines, aldehydes, nitriles, ketones, hydrocarbons, and alcohols.

Through careful visual inspection of the profiles and statistical comparison of integrated peak areas of the urinary volatiles obtained from six experimental groups of animals, we established that 13 constituents (including two unidentified substances) showed obvious variations in their chromatographic peak areas (Table 1). The remaining substances listed in this table demonstrated relatively constant concentration in the urine samples of all investigated animals.

Study 1: Volatile Profiles of Female Pine Vole Urine. A quantitative comparison of urinary profiles from unexposed and exposed females revealed that exposure depresses the level of certain volatile compounds (Figure 2). Pyrazines (peaks 3 and 6) were absent in the urine of exposed females. A significant ($P < 0.01$) decrease in concentration was observed with phenylacetonitrile (peak 16) and three ketones (peaks 14, 15, and 19). Statistical analysis revealed that only two compounds, nonanal (peak 8) and an unidentified substance (peak 22), significantly ($P < 0.01$) increased their concentration after exposure (Figure 2).

Figure 3 (A-F) displays plots for concentrations of selected compounds at various times after estrogen implantation. The selected compounds were those that exhibited fluctuation in concentration compared to the control values obtained from intact females (01 h after implantation). Ovariectomy (02 h after implantation) significantly ($P < 0.01$) depressed the concentration of some urinary volatiles when compared to intact values. These compounds were pyrazines (peaks 3 and 6), phenylacetone (peak 14), γ -octanoic lactone (peak 17),

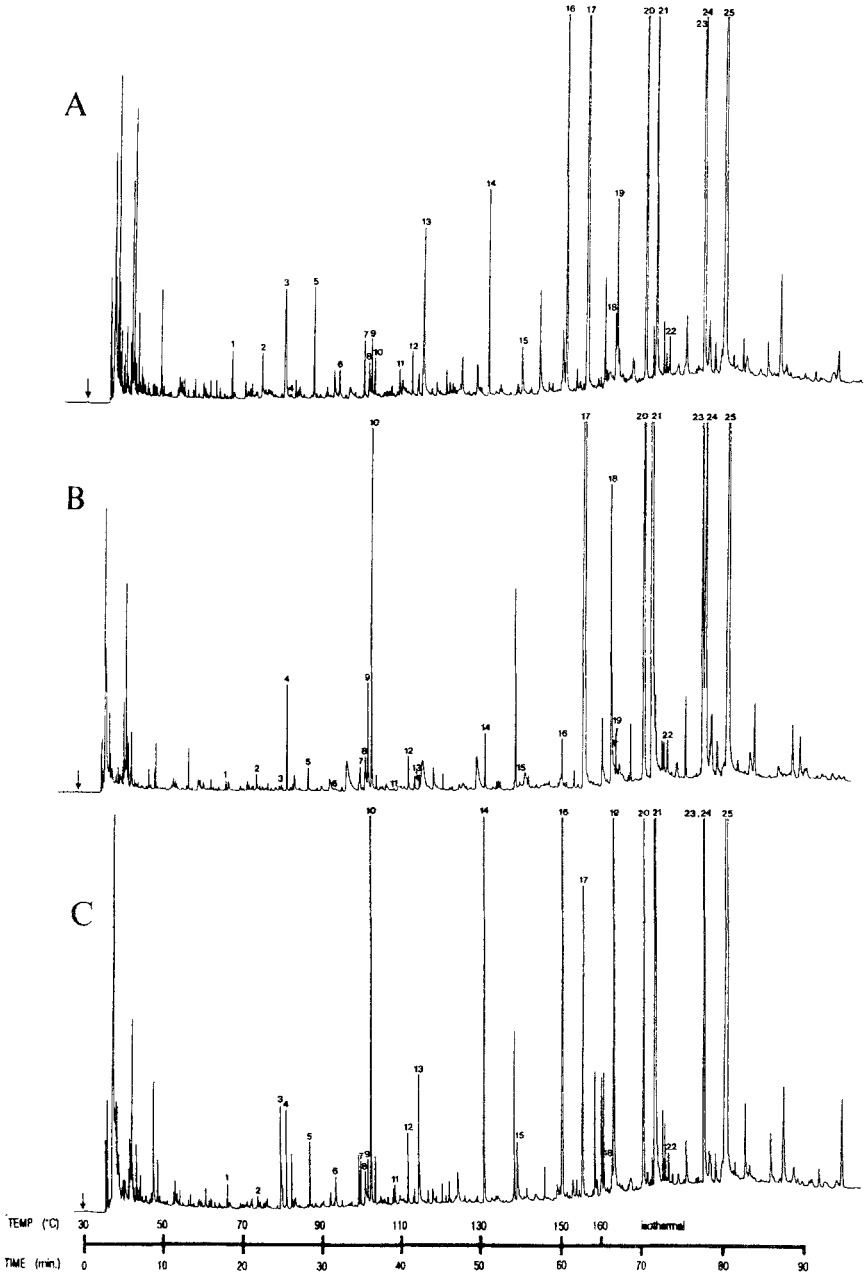


FIG. 1. Gas-chromatographic profiles of volatile constituents from urine of (A) unexposed female; (B) female exposed to male-soiled bedding, and (C) intact male pine voles.

TABLE 1. VOLATILE CONSTITUENTS OF FEMALE AND MALE PINE VOLE URINE IDENTIFIED FROM CHROMATOGRAMS IN FIGURE 1

Peak number	Structural identification	Significant concentration changes
1	2-heptanone	
2	limonene	
3	2,5-dimethylpyrazine	+
4	<i>p</i> -cymene	
5	6-methyl-5-hepten-2-one	
6	2,3,5-trimethylpyrazine	+
7	2-nonanone	
8	nonanal	+
9	benzaldehyde	+
10	<i>p</i> -methylpropenylbenzene	+
11	5-methylfurfural	
12	benzonitrile	+
13	unidentified	+
14	phenylacetone	+
15	3-decen-2-one	+
16	phenylacetonitrile	+
17	γ -octanoic lactone	+
18	phenol	
19	geranylacetone	+
20	γ -nonanoic lactone	
21	<i>p</i> -cresol	
22	unidentified	+
23	<i>p</i> -ethylphenol	
24	γ -decanoic lactone	
25	δ -decanoic lactone	

and an unidentified compound (peak 22). Only peak 13 (unidentified) increased ($P < 0.01$) in concentration after removal of the ovary.

Urine from estrogen-treated females collected at 48–72 hr after implantation contained compounds such as ketones (peaks 14, 15, and 19; Figure 3A, B), pyrazines (peaks 3 and 6; Figure 3C), and nitriles (peaks 12 and 16; Figure 3D) in considerably lower concentration than did intact and ovariectomized animals. Urinary profiles of the females implanted with estrogen for 48–72 hr tended to mimic the profile of exposed animals. At 144 hr postimplantation, all these compounds returned toward the control, intact values (Figure 3A–D). Only the γ -octanoic lactone (peak 17) exhibited quick return to the intact level within 48 hr after estrogen implantation. This lactone exhibited the greatest fluctuation in concentration throughout the estrogen test period. As seen in Fig-

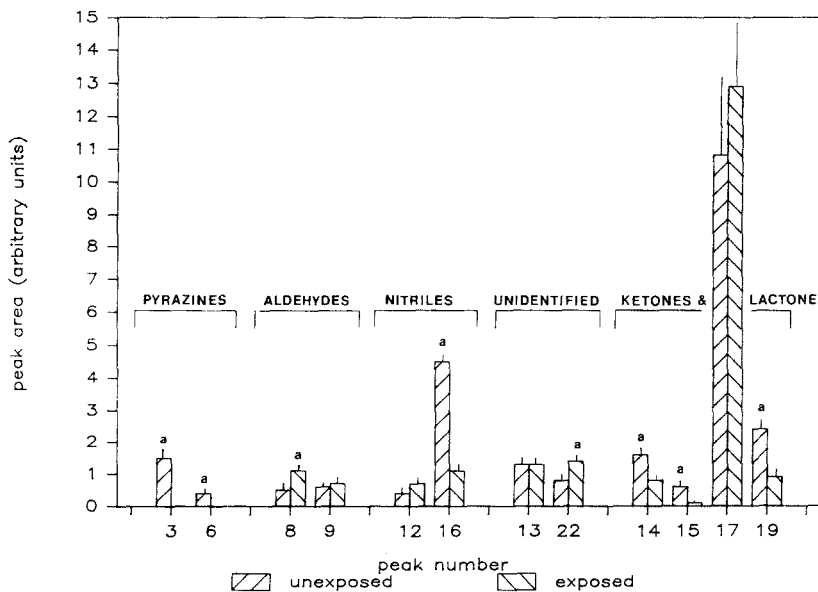
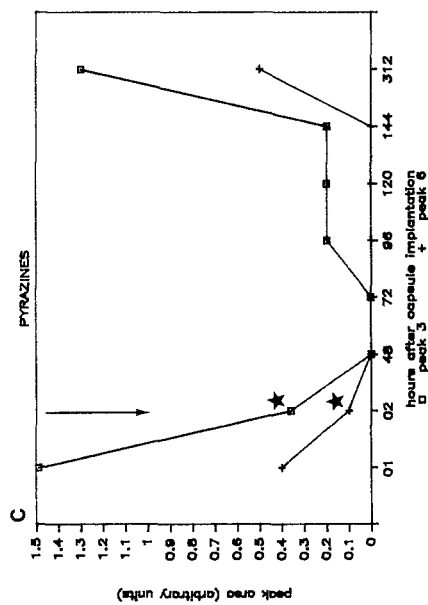
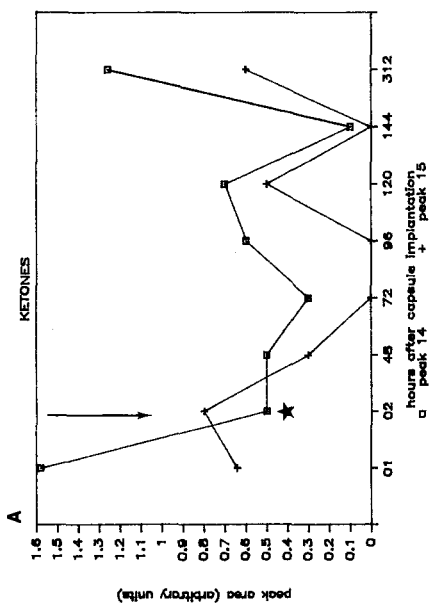
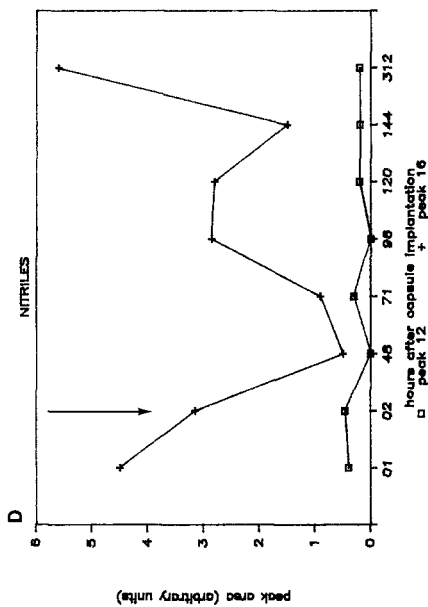
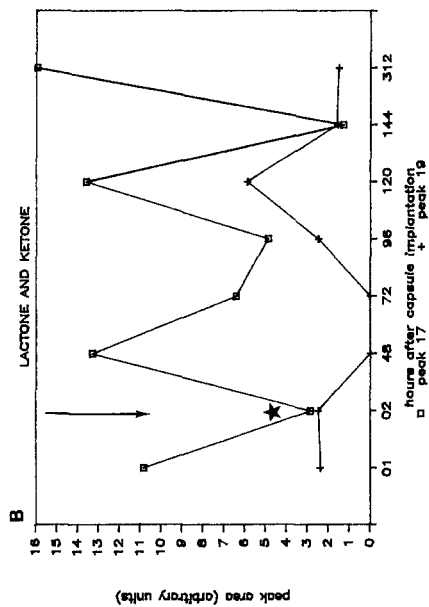


FIG. 2. Urinary constituents exhibiting significant changes in the peak areas (\pm SEM) between unexposed and exposed female pine voles. Letter a indicates 0.01 level of significance over unmarked bars.

ure 3E, aldehyde concentrations were changed during estrogen implantation. Nonanal and benzaldehyde (peaks 8 and 9) displayed two separate periods of increase after estrogen implantation, the first at 48 hr for both compounds and the second at 96 hr for benzaldehyde and at 120 hr for nonanal. In the 312-hr postimplantation period, concentrations of these aldehydes were found near the control levels of intact animals. Nonanal also displayed elevated concentration after exposure to male-soiled bedding (Figure 2).

Two unidentified compounds (peaks 13 and 22) displayed different behavior after estrogen treatment when compared to identified compounds (Figure 3F). Peak 13 decreased dramatically within 48 hr after estrogen implantation and was not present in the urine for the next five collection periods. This compound appeared again at a low level at 312 hr after implantation. The level of compound 22 rose gradually from 48 hr to 72 hr after treatment, and decreased to zero level at 120 hr. The presence of this compound was detected again at 312 hr after estrogen implantation, but still at a lower level than for the controls.

Study 2: Volatile Profiles of Male Pine Vole Urine. The profile of castrate male urine was very similar to that of intact male urine. Quantitative comparisons showed that only the phenylacetone level (peak 14) was significantly higher



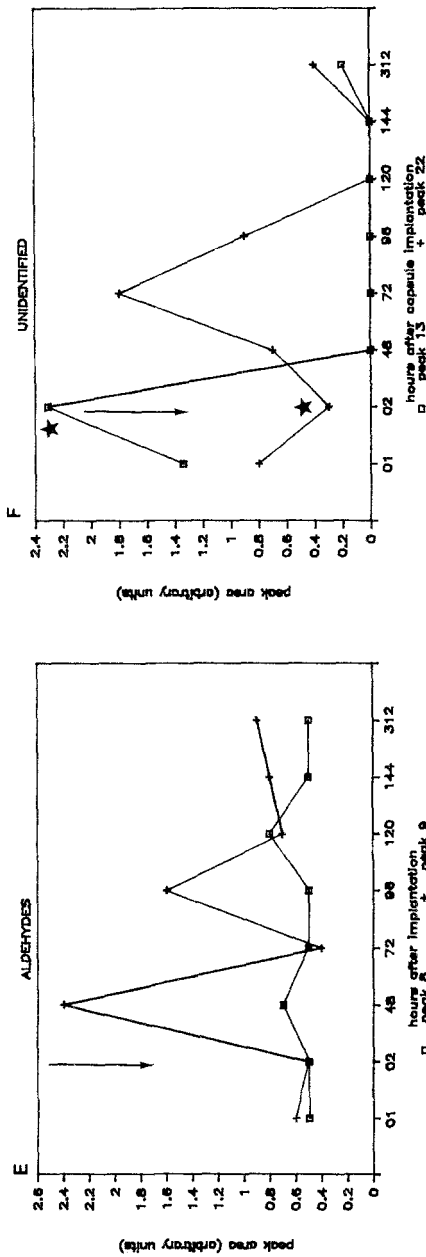


FIG. 3. Relative effect of estrogen treatment on urinary volatiles in adult pine vole females. The control conditions: intact and ovariectomized values are represented at 01 h and 02 h after implantation. The peak area (arbitrary units) of selected compounds at various times after estrogen implantation are presented in the following groups: A, ketones; B, lactone and ketone; C, pyrazines; D, nitriles; E, aldehydes; and F, unidentified. The arrows indicate the time of capsule implantation. The asterisks indicate a significant ($P < 0.01$) difference between peak areas (\pm SEM) of intact and ovariectomized females.

in intact male urine than in castrate urine (1.4 ± 0.3 vs. 0.5 ± 0.1 arbitrary units; $P < 0.05$).

A quantitative comparison of the urinary volatiles of intact males and intact females showed four compounds at significantly ($P < 0.05$) different levels (Figure 4). Peaks 3, 6, and 17 (2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and γ -octanoic lactone) were significantly higher in female urinary samples as compared to the male samples. Only one compound, *p*-methylpropenylbenzene (peak 10), was significantly higher in the male urine in comparison to the female urine. No compounds typical to either the female or male were observed.

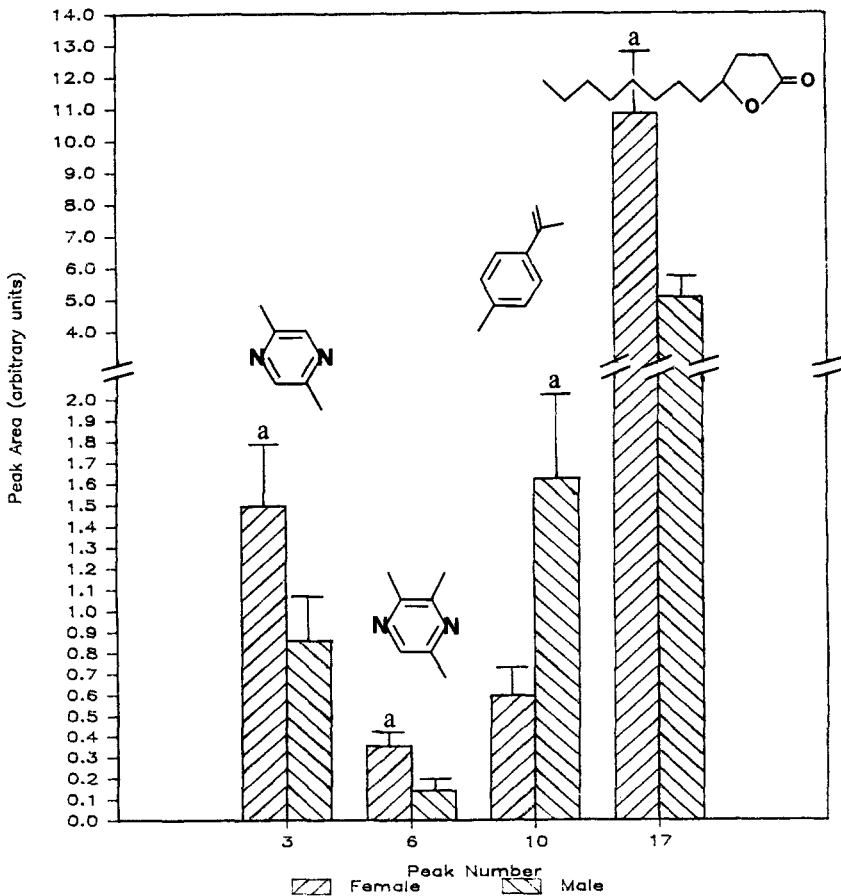


FIG. 4. Sex-dependent changes in peak areas (\pm SEM) of urinary volatiles from pine vole, *Microtus pinetorum*. Lettered bars (a) indicate significant changes ($P < 0.05$) over unmarked bars.

DISCUSSION

A typical gas-chromatographic profile of pine vole urine contains relatively few volatile constituents (around 30) as compared with the volatile-rich house mouse urine (well over 100 constituents; Schwende et al., 1986).

Many of the compounds present in pine vole urine have also been recognized as mouse urinary volatiles (Schwende et al., 1986; Andreolini et al., 1987; Jemiolo et al., 1987). Benzaldehyde, *p*-cresol, phenol, phenylacetone, and 6-methyl-5-hepten-2-one are common to both vole and house mouse urine. However, some urinary constituents appear to be typical to the vole. These compounds are γ -octanoic lactone, γ -nonanoic lactone, γ -decanoic lactone, and δ -decanoic lactone.

In female pine voles, 10 identified volatiles demonstrated significant changes in concentration after hormonal manipulation. Ovariectomy depresses the concentration of pyrazines, phenylacetone and γ -octanoic lactone when compared to the levels recorded for intact animals. Twenty-four-hour exposure to male-soiled bedding or 48- to 96-hr estrogen treatment of mature female pine voles also tended to depress the concentration of selected compounds in great proportion when compared to intact animals. Both male-soiled bedding and estrogen implantation provoke an increase in concentration of only two compounds: nonanal (peak 8) and an unidentified substance (peak 22). The similarities in appearance of volatile profiles from exposed females to these from estrogen-treated animals can be attributed to the elevated estrogen levels in exposed females triggered by male chemosignals. It has been shown that chemical cues from male urine may trigger the hormonal system, leading to secretion of estrogen and reproductive activation in immature and adult virgin female voles (Carter et al., 1980; Baddaloo and Clulow, 1981; Petersen, 1986).

Urine from female voles (Getz et al., 1983; Frankiewicz and Marchlewska-Koj, 1985), similar to that from the house mouse (Drickamer, 1977), features a chemical signal that suppresses reproductive maturation in other females. We have previously established that 2,5-dimethylpyrazine present in the urine of grouped female mice delays puberty in female conspecifics (Novotny et al., 1986). Results of the present study reveal that urine of *Microtus pine-torum* also contains two pyrazine derivatives: 2,5-dimethylpyrazine and 2,3,5-trimethylpyrazine. Ovariectomy significantly depressed concentration of both compounds. Exposure and estrogen implantation abolished their presence in the urine. In mice, ovariectomy does not change the puberty-delaying biological activity of urine. Urine from ovariectomized animals is equally as effective in delaying puberty as intact urine (Drickamer et al., 1978). In contrast to *Mus musculus*, pine voles are induced ovulators (Schadler and Butterstein, 1979). The reproductive system of female voles remains in a quiescent state in the absence of stimulation from males. Induced ovulation arises as a direct result

of the acute initiation of a surge of LH secretion by external (sexual) stimuli. An LH surge initiated by endogenous mechanisms involving endocrine feedback is typical for spontaneous ovulators such as the house mouse. The different hormonal environment of female pine voles can thus have a different influence on the levels of metabolites excreted in the urine as compared to the house mouse.

Castration of males in both *Mus musculus* (Bruce, 1965), and *M. ochrogaster* (Carter et al., 1980) reduces the stimulatory potency of their urinary chemosignals. In the present study, however, only one urinary volatile exhibited significant quantitative differences after castration. This compound, phenylacetone, has also been observed in the urine of several other mammals (Schwende et al., 1986; Raymer et al., 1986). The lack of differences between intact and castrate urinary volatiles may indicate that chemosignals of male pine voles are either not dependent upon testosterone levels or that they are nonvolatile substances and, therefore, not observed in the volatile profile. Lepri and Vandenberg (1986) reported that intact and castrate urine were equally effective in eliciting female reproductive activation.

There are no qualitative differences between urinary profiles of male and female pine voles. It is, however, worthwhile to note that female urine contains three volatiles in higher concentration than male urine: γ -octanoic lactone (ovariectomy decreased its level) and two pyrazines (structurally similar to the puberty delay factor in female mouse urine). Male vole urine contained only one compound (*p*-methylpropenylbenzene) that was present in higher concentration when compared with female urine. Its concentration, however, was not changed after castration.

This paper describes, for the first time, a chemical analysis of urinary volatiles in pine voles. Several lactone derivatives were found as the typical constituents of this species. In contrast to the previous investigation of the house mouse by this laboratory, no drastic quantitative differences were found due to a variety of endocrinological manipulations, although statistically significant excretion changes were noticed. Future inquiries into the role of chemicals in the reproduction of the pine vole may need to concentrate on the nonvolatile urinary fraction.

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ECOLOGICAL ASPECTS OF CACTUS TRITERPENE GLYCOSIDES I. THEIR EFFECT ON FITNESS COMPONENTS OF *Drosophila mojavensis*

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Abstract—The effects of pentacyclic triterpene glycosides extracted from agria and organ pipe cacti on three fitness parameters of the cactophilic fruit fly, *Drosophila mojavensis* were tested. Triterpene glycosides from organ pipe increased development time and reduced larval viability while those from agria produced smaller adults (reduced fecundity). In addition, the microbial communities in the organ pipe saponin media were less dense than those in the media to which agria saponins had been added. The role of cactus triterpene glycosides in the ecology of this *Drosophila* species is discussed.

Key Words—*Drosophila*, Diptera, Drosophilidae, triterpene glycosides, cactus, fitness components, host-plant relationships.

INTRODUCTION

One of the most interesting classes of natural organic compounds is the triterpenoidal saponins (triterpene glycosides). These compounds have comparatively large molecular weights and high polarity, are structurally diverse, and are widely distributed among members of the plant kingdom. The interest in these compounds stems from the incredible variety of biological activities that they reportedly exhibit. In addition to the characteristic foaming when aqueous solutions are shaken, saponins are hemolytic and have been used as herbal drugs with expectorant, antitussive, tonic, and diuretic activities. Certain purified saponins have been demonstrated to be antiinflammatory, work on the central

nervous system, prevent stress ulcers, have antifatigue activity, be antibacterial and antifungal agents, stimulate lipid metabolism, promote nucleic acid and protein synthesis, have antitumor or anticancer effects, and kill fish and some insects (Natori et al., 1981; Chauhan and Srivastava, 1978; Chandel and Rastogi, 1980). Many of the biological effects of the famous Oriental herb drug, ginseng root, are attributed to the saponins that are present in this plant (Chandel and Rastogi, 1980). The biological activity is not necessarily the sole property of the sapogenin (aglycone) portion of these molecules since recent studies of saponins isolated from Oriental drugs have discovered that saponins consisting of the same sapogenin, but with different sugar sequences, have different biological activities (Natori et al., 1981).

Investigations into the triterpenes of columnar cacti began in the 1950s with the work of Carl Djerassi and his colleagues. They reported the presence of glycosides of oleanolic acid and thurberogenin in organ pipe cactus (Djerassi et al., 1953; Marx et al., 1967) and glycosides of gummosogenin, machaeric acid, and machaerinic acid in agria cactus (Djerassi et al., 1954, Djerassi and Lippman, 1955). In the 1970s, William Heed and Henry Kircher joined in the study of cactus saponins, prompted by their suspicion that these compounds played a role in *Drosophila* ecology. Organ pipe cactus (*Stenocereus thurberi*) and a related species, agria cactus (*S. gummosus*), are endemic to the Sonoran Desert of North America, and necrotic stems of both cacti are used as feeding and breeding substrates of the cactophilic fruit fly, *Drosophila mojavensis* (Heed, 1978). Kircher (1977, 1980) identified a third pentacyclic triterpene, queretaroic acid, in the glycoside fraction of organ pipe. He also reported that the concentration of triterpene glycosides in organ pipe stems decreases from the surface to the inner part of the plant and that glucose and rhamnose are two of the constituent sugars. It was suggested that the concentration gradient of triterpene glycosides may reflect their role as a protective agent against herbivores and/or for osmotic protection against freezing.

Subsequent studies (reported in Kircher, 1982) of the saponins of agria and organ pipe have demonstrated their relatively high concentration and contributed some structural information. On a dry weight basis, the tissue averages of saponins for organ pipe and agria are 28% and 36%, respectively. Each of the triterpenes in organ pipe is attached to a tetrasaccharide consisting of two glucose and two rhamnose residues. The structural relationships among agria triterpene glycosides is not as well understood. A preliminary study involving the hydrolysis of agria triterpene glycosides resulted in an approximate 2:1 ratio of glucose to rhamnose and a 4:1 ratio of sugars to aglycones.

Information on the role of triterpene glycosides in *Drosophila* ecology has been rather fragmentary. The fact that *D. nigrospiracula*, another cactophilic fly, does not typically use agria and organ pipe rots has been known since the

early 1970s (Fellows and Heed, 1972). One of the major chemical differences between these cacti and the typical host plants for *D. nigrospiracula* (saguaro, *Carnegiea gigantea*; and cardon, *Pachycereus pringlei*) is the presence of triterpene glycosides. For this reason, agria and organ pipe saponins were thought to be involved in determining host plant utilization patterns by the desert-adapted flies. Kircher (1977) first reported that the addition of organ pipe triterpene glycosides to the medium inhibited the development of *D. nigrospiracula* larvae. Subsequent studies, using first-instar larvae rather than adults as the starting material, failed to substantiate the previous study and, instead, pointed to several other compounds as being the primary determinants in excluding *D. nigrospiracula* from agria and organ pipe necroses (Fogleman et al., 1986). However, a statistically significant reduction in the larval viability of *D. mojavensis* in the presence of organ pipe triterpene glycosides was demonstrated.

Several other ecological aspects of agria and organ pipe cacti and their resident drosophilids are noteworthy. First, the geographic distributions of these two species of cacti are different. Organ pipe occurs on both the Baja peninsula and on the mainland (Sonora, Mexico), while agria is essentially limited to the peninsula (Heed, 1978). The populations of *D. mojavensis* in the two regions are genetically differentiated and partially reproductively isolated (Heed, 1978; Zouros and d'Entremont, 1980). *Drosophila mojavensis* on the peninsula primarily use agria rots as substrates even though organ pipe cacti are present, and, finally, the thorax lengths of wild-caught *D. mojavensis* females from agria in Baja are smaller than those from organ pipe on the mainland (0.8–0.9 mm vs. approx. 1.0 mm, respectively; Mangan, 1982). Thorax length in flies is a fitness parameter that is positively correlated to fecundity (Mangan, 1978) and migratory ability (Roff, 1977).

In an extensive set of experiments, Etges and Heed (1987) investigated the variation in three fitness components, egg-to-adult viability, development time, and adult thorax size, between Baja and mainland populations of *D. mojavensis* as adaptive responses to the host plant shift from agria to organ pipe. They found that organ pipe rots were qualitatively poorer substrates than agria rots for both populations, especially at higher larval densities. Using organ pipe as a substrate caused longer egg-to-adult development times, lower viabilities, and smaller thorax sizes than agria. But since whole tissue was used, these experiments do not specifically address the question of which chemical constituents of the cacti are responsible for the observed variation in fitness components. The experiments reported herein were designed to test the effect of agria and organ pipe saponins on fitness parameters in *D. mojavensis* in order to clarify their role in producing differences in these fitness components. This information should contribute to our understanding of the adaptation of *D. mojavensis* to agria and organ pipe as it relates to host plant utilization.

METHODS and MATERIALS

Triterpene Glycoside Extraction. Organ pipe and agria triterpene glycosides were extracted from fresh tissue as follows: cactus tissue (without the skin and the woody core) was homogenized in a blender with an equal volume of methanol. The homogenate was filtered through a Buchner funnel, and the residue was exhaustively extracted with 2:1 chloroform-methanol solvent in a continuous Soxhlet extractor. The original methanol and the chloroform-methanol filtrates were combined, evaporated to dryness, and distributed between water and ether in a large separatory funnel. The aqueous phase was removed and extracted with *n*-butanol. Purified triterpene glycosides were obtained by concentration of the butanol via a rotary evaporator followed by drying in a 50°C oven. Several kilograms of tissue from each cactus species were used in order to insure that a sufficient amount of triterpene glycosides was obtained. Purity of the glycosides was checked by thin-layer chromatography.

Test Media. Media for testing the effect of triterpene glycosides on larval viability, developmental rate, and thorax length were prepared by adding the glycosides to homogenized saguaro tissue that had been previously rotted. Saguaro was used as the basic substrate because its secondary chemistry does not include triterpene glycosides or any other compounds at concentrations that would affect the larval viability of *D. mojavensis* (Kircher, 1982). In addition, the use of saguaro provides a substrate that is much closer to natural agria and organ pipe rots with respect to substrate consistency and microorganismic growth characteristics than artificial media. The test substrate was prepared by homogenizing 2 k of fresh saguaro tissue and inoculating it with tissue and rot liquid from an established rot. After 10 days of incubation, the saguaro rot was divided into five batches of 300 g each and triterpene glycosides (TTGs) were added as follows: no TTGs (control), 13.5 g of agria TTGs, 18.0 g of agria TTGs, 13.5 g of organ pipe TTGs and 18.0 g of organ pipe TTGs. The quantities, 13.5 and 18.0 g, represent 30% and 40% dry weight, respectively, which are close to the published concentrations of 28% dry weight in organ pipe and 36% in agria (Kircher, 1982). In all cases, the resulting mixture was blended to ensure homogeneity and distributed into 12 Petri plates (25 g of homogenate per plate). Each plate was then inoculated with 1 ml of a suspension of six species of cactophilic yeasts commonly isolated from natural rots of both agria and organ pipe. The suspension was made by harvesting 48-hr cultures of each species. These yeasts were originally obtained from Dr. Tom Starmer (Syracuse University) and had been kept in lab culture for several years. The yeast species (with Syracuse University identification numbers) were *Pichia cactophila* (78-32), *P. mexicana* (79-289.2), *P. amethionina* var. *amethionina* (81-265.1), *Candida sonorensis* (80-334.4), *C. ingens* (81-208.3), and *Cryptococcus cereanus* (80-322.6). All

plates were incubated for three days in order to allow the yeasts to reach a reasonable density.

Drosophila mojavensis Larvae. A multifemale strain of *D. mojavensis* was established from approximately 50 males and females collected from natural agria rots at Punta Onah, Sonora, Mexico, in December 1985. Agria and organ pipe cacti are sympatric in this area. The strain had been maintained in the laboratory on Carolina instant *Drosophila* food (Formula 4-24) for six months. First-instar larvae were obtained by allowing adults to oviposit on water-agar Petri plates which contained a 2.5 cm patch of paste made from bakers' yeast and water. The paste stimulates oviposition. Adults were transferred to new plates on a daily basis, and eggs were given 24 hr to hatch. First-instar larvae were, therefore, 24 hr old or less. A small probe was used to transfer the larvae from the oviposition plates onto a 2.5 cm square of paper towel in the center of each plate of test medium. Two different larval densities, 50 larvae per plate and 400 larvae per plate, were used. These densities were chosen so that comparisons could be made with previous experiments (Etges and Heed, 1987). Six replicates of each test medium were produced. A replicate series, then, consisted of 10 Petri plates: 50 larvae-no TTGs, 50 larvae-30% agria TTGs, 50 larvae-30% organ pipe TTGs, 50 larvae-40% agria TTGs, 50 larvae-40% organ pipe TTGs, 400 larvae-no TTGs, 400 larvae-30% agria TTGs, 400 larvae-30% organ pipe TTGs, 400 larvae-40% agria TTGs, and 400 larvae-40% organ pipe TTGs. Each replicate series was set up using larvae from the same collection batch. All experiments were performed at ambient temperature and humidity. These conditions varied slightly during the experiments, but all plates were exposed to the same environmental conditions.

Microbial Density Determinations. Replicate series numbers 3, 4, and 5 were sampled one week after the introduction of larvae. A small amount (0.1 g) of the medium from each plate was taken and examined to ensure that no larvae were inadvertently included. These samples were serially diluted, and aliquots of the dilutions tubes were plated on both acidified yeast extract-malt extract agar (AYMA) and nutrient agar (NA) containing antifungal chemicals. AYMA is a general yeast medium that supports the growth of all cactophilic yeasts. It was acidified to pH 3.8 with 1 N H_3PO_4 in order to inhibit the growth of bacteria. Nutrient agar is a general growth medium for bacteria. Cycloheximide (100 $\mu\text{g}/\text{ml}$) and amphotericin B (10 $\mu\text{g}/\text{ml}$) were added to the NA to inhibit the growth of yeasts. The number of colonies that appeared on each plate was recorded and used to calculate the colony forming units (CFUs) per gram of test substrate.

Data Collection and Analysis. The number of adults that eclosed from each plate was recorded daily until eclosion ceased. The total for each plate divided by the number of larvae that were introduced (multiplied by 100) represents the

percent viability (larva to adult). Mean developmental rate was calculated as the average of the frequency distribution relating the number of flies that eclosed (Y axis) to the number of days after the start of the experiment (X axis). Thorax length was measured on a random sample of five males and five females eclosing during the first three days of the eclosion period for each plate. Data were analyzed using two-way analysis of variance tests (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

The means and standard deviations of the fitness parameters that were measured are presented in Table 1, and the statistical analyses of these data are given in Table 2. In general, the results of the two-way analyses of variance were the same for all of the fitness parameters: high significance ($P < 0.01$) with respect to treatments (triterpene glycoside type and concentration) and larval density and no significant interaction. Each fitness parameter will now be discussed in turn.

Estimates of larval viability of *D. mojavensis* under various conditions have appeared in several recent reports (Fogleman, 1984; Fogleman et al., 1986; Etges and Heed, 1987). Different substrates (agria, organ pipe, and saguaro) and different larval densities are known to influence this parameter. The control data for larval viability in saguaro homogenate given in Table 1 are comparable to the average viabilities of cactophilic *Drosophila* species in their typical cactus substrate, which was reported as about 75% (Fogleman, 1984). From the statistical analysis, substrate treatment and larval density were about equal in significance. At a larval density of 50 per replicate, the two concentrations of organ pipe triterpene glycosides resulted in the lowest average percent viabilities. At the higher larval density of 400 per replicate, 40% organ pipe saponins again produced the lowest viability. Thus, the glycosides of organ pipe appear to be more effective than those of agria. In all cases, the addition of triterpene glycosides to the medium resulted in lower viability, and the greater the concentration, the lower the parameter value.

All other parameters being equal, development time is negatively correlated with fitness in *Drosophila* (Lewontin, 1965). The statistical analysis of the larva-to-adult development rate shows that, although both treatment and larval density are significant sources of variation in this parameter, larval density produces the major effect ($F = 4.297$, $P < 0.01$ vs. $F = 182.550$, $P \ll 0.001$ for treatments and larval density, respectively). The pattern of the effects produced by the various triterpene glycosides and larval densities is, however, identical to that for larval viability. That is, at 50 larvae per replicate, the two concentrations of organ pipe saponins resulted in the longest development times and, therefore, the lowest fitness. At 400 larvae per replicate, the longest devel-

TABLE 1. EFFECT OF TRITERPENE GLYCOSIDES (TTGs) ON FITNESS PARAMETERS OF *D. mojavensis*^a

Treatment ^b	Viability (%)	Developmental rate (in days)	Thorax length (mm)	
			Females	Males
50 larvae per replicate				
Control	78.7 ± 9.9	13.7 ± 0.4	1.11 ± .014	1.03 ± .023
30% AG TTGs	78.0 ± 12.8	14.2 ± 0.6	1.07 ± .030	0.99 ± .015
40% AG TTGs	70.3 ± 26.2	14.1 ± 0.8	1.06 ± .016	0.98 ± .035
30% OP TTGs	64.7 ± 17.0	14.4 ± 1.1	1.10 ± .022	1.00 ± .012
40% OP TTGs	49.0 ± 25.6	15.7 ± 1.9	1.07 ± .036	1.00 ± .032
400 larvae per replicate				
Control	76.2 ± 5.5	17.8 ± 0.7	1.00 ± .035	0.94 ± .033
30% AG TTGs	61.5 ± 11.8	18.1 ± 0.7	0.96 ± .032	0.89 ± .025
40% AG TTGs	50.0 ± 23.1	19.0 ± 1.6	0.96 ± .029	0.87 ± .066
30% OP TTGs	63.0 ± 13.8	17.5 ± 1.2	1.01 ± .027	0.92 ± .033
40% OP TTGs	43.8 ± 16.4	19.1 ± 1.2	1.02 ± .021	0.94 ± .030

^aMeans ± standard deviations for six replicates are reported for two different larval densities.^bAG = agria; OP = organ pipe.

opment times were measured in the two 40% concentration treatments regardless of the source of saponins, but the addition of organ pipe triterpene glycosides at 40% dry weight resulted in the longest development time. With one exception (30% organ pipe TTGs), addition of triterpene glycosides increased the larva-to-adult development time and lowered fitness.

One of the goals of these experiments was to clarify the role of cactus triterpene glycosides in producing the observed differences in thorax sizes of *D. mojavensis* using agria on the Baja Peninsula and those using organ pipe on the mainland. Analysis of treatment effects on thorax lengths (Table 1) were performed keeping sexes separate since female *Drosophila* are typically larger than males. The data clearly demonstrate that, while larval exposure to organ pipe triterpene glycosides results in flies with thorax sizes similar to those from control substrates, agria triterpene glycosides make flies noticeably smaller. Since the 30% organ pipe and 40% agria TTG treatments are closest to the average tissue values for these cacti (Kircher, 1982), mean thorax sizes from these treatments were statistically compared to means from the controls using the Student-Newman-Keuls (SNK) procedure (Sokal and Rohlf, 1981). The results are given below (underlining connects treatments whose means are not significantly different at the 0.05 level):

Females, 50 larvae/replicate: Agria < Organ pipe Control

Females, 400 larvae/replicate: Agria < Organ pipe Control

Males, 50 larvae/replicate: Agria Organ pipe Control

Males, 400 larvae/replicate: Agria Organ pipe Control

The reduction in thorax length by agria triterpene glycosides was more significant in females than in males, but, in all of the above cases, the agria saponins produced significantly smaller flies as compared to controls. The females that eclosed from the agria treatments were also significantly smaller than the females from the organ pipe treatments.

Measuring the thorax size of the flies that were first to emerge might be misleading if thorax size changes as a function of development time. In order to check on this phenomenon, thorax measurements were taken on a smaller sample of flies that emerged approximately one week after the initial group. Although the differences in thorax lengths of flies emerging from the organ pipe TTG medium as compared to the agria TTG medium were reduced in this sample, the overall conclusions regarding the effect of triterpene glycosides on thorax sizes remained unchanged.

The statistical analyses of thorax sizes (Table 2) exhibit the same patterns as the previous analyses in that larval density is the major source of variation.

TABLE 2. RESULTS OF ANALYSIS OF VARIANCE TESTS^a

Parameter	Source of variation			
	Treatment	Larval density	Interaction	Error
Viability (%)				
Degrees of freedom	4	1	4	50
Sum of squares	6451	1276	884	15278
F value	5.278	4.176	0.723	
Probability	<0.01	<0.01	not sig.	
Development Time				
Degrees of freedom	4	1	4	50
Sum of squares	21.0	222.7	6.4	61.0
F value	4.297	182.550	1.313	
Probability	<0.01	<<0.001	not sig.	
Thorax length of females				
Degrees of freedom	4	1	4	40
Sum of squares	0.021	0.103	0.006	0.031
F value	6.894	134.868	1.989	
Probability	<0.001	<<0.001	not sig.	
Thorax length of males				
Degrees of freedom	4	1	4	50
Sum of squares	0.026	0.118	0.003	0.059
F value	5.435	100.095	0.681	
Probability	<0.01	<<0.001	not sig.	
Bacterial growth				
Degrees of freedom	4	1	4	20
Sum of squares	7.352	0.226	0.754	1.769
F value	20.782	2.573	2.132	
Probability	<0.001	not sig.	not sig.	

^aThe effects of triterpene glycosides (TTGs) from *agria* and organ pipe cactus (treatment) on larva-to-adult viability, development time, female thorax length, male thorax length, and bacterial growth at two different larval densities are indicated. Data on bacterial growth were log₁₀-transformed prior to analysis.

However, with respect to thorax size, it is the *agria* saponins, rather than those of organ pipe, that reduce fitness.

Etges and Heed (1987) concluded that organ pipe is a nutritionally poorer substrate than *agria* and suggested that this may be partly due to differences in the density of the microbial communities residing in rots of the two cactus species. Microbes are known to be essential components of the ecology of *D. mojavensis* since sterilized organ pipe tissue does not support the development of axenic larvae (Starmer, 1982). *Drosophila mojavensis* larvae (from sterilized eggs) are also not viable on fresh *agria* tissue (Etges and Heed, 1987). Determinations of yeast community density (cells per gram) from naturally occurring

agria and organ pipe rots show a large amount of variation between samples even within a species (Fogleman and Starmer, 1985). The largest yeast densities, however, have been measured in agria.

The results of the experiment on the effect of triterpene glycosides from agria and organ pipe on the growth of yeasts and bacteria are given in Table 3. The data on yeast density were not statistically analyzed as before due to the large number of samples in which the density was below the limits of detection. The detection limit was 1000 cells/g of tissue (a \log_{10} of 3.000) due to the dilution series that was employed. The dilutions that were plated were chosen primarily because previous experience suggested that the densities would be considerably higher than the detection limit, and, therefore, the lower dilutions were not plated. In general, yeast growth in the control substrate and in the substrates with organ pipe triterpene glycosides was minimal. Addition of agria saponins promoted the growth of the yeast community. Previous experiments

TABLE 3. EFFECT OF TRITERPENE GLYCOSIDES (TTGs) ON MICROBIAL GROWTH ^a

Treatment	No.	50 Larvae		400 Larvae	
		Yeast	Bacteria	Yeast	Bacteria
Control	3	<3.000	8.613	<3.000	8.724
	4	3.301	8.531	<3.000	9.258
	5	3.000	8.925	<3.000	9.342
	Average	<3.000	8.690	<3.000	9.180
30% Agria TTGs	3	5.000	9.602	4.716	9.663
	4	6.898	9.763	5.079	9.362
	5	6.279	9.367	5.352	8.477
	Average	6.059	9.577	5.049	9.167
40% Agria TTGs	3	4.699	9.461	4.613	9.021
	4	4.531	9.537	3.845	9.316
	5	3.544	9.079	3.903	9.379
	Average	4.258	9.359	4.120	9.239
30% Organ Pipe TTGs	3	<3.000	9.515	3.301	8.857
	4	4.000	9.212	<3.000	8.851
	5	4.255	8.869	<3.000	8.949
	Average	<3.752	9.199	<3.000	8.886
40% Organ Pipe TTGs	3	<3.000	8.243	<3.000	7.601
	4	<3.000	8.079	<3.000	7.591
	5	<3.000	8.322	<3.000	8.114
	Average	<3.000	8.215	<3.000	7.769

^aThe figures presented represent the logarithm of the colony forming units (CFUs) per gram of necrotic tissue. Counts were taken seven days after the introduction of larvae.

have demonstrated that some cactophilic yeasts can use cactus triterpene glycosides as a carbon source (Williams, 1985) while others (not included in this study) are definitely inhibited by the presence of cactus triterpene glycosides (Starmer et al., 1980; Phaff et al., 1985). The most likely explanation for the low cell density of yeasts in the control substrate is the absence of triterpene glycosides and the relatively low concentration of other usable carbohydrates (Kircher and Al-Azawi, 1985). Differences in the response to the saponins of the two cactus species may be related to the differences in their chemical structures, particularly with respect to the carbohydrate moieties. The fact that yeast density was significantly greater in the 30% agria TTG treatment than in the 40% agria TTG treatment ($F = 13.876$; $df = 1,8$; $P < 0.01$) demonstrates the complexity of the interaction between cactus saponins and yeasts. Further experimentation is necessary to fully elucidate these relationships.

Table 3 also contains the results of the determinations of bacterial densities in the experimental media. The statistical analysis of these data appears in Table 2. When all of the data are analyzed together, the treatment effect (TTGs) was the only significant source of variation in the data, and the bacterial communities in media containing organ pipe saponins were noticeably less dense. When the data from the organ pipe treatments are analyzed by themselves, however, larval density is also shown to significantly reduce the bacterial concentration ($F = 8.140$; $df = 1,8$; $P < 0.01$). The overall conclusion from this portion of the experiments is that triterpene glycosides do influence microbial communities in that both yeasts and bacteria are reduced in density when exposed to organ pipe saponins as compared to those of agria. Since these microbes are nutritional components for *Drosophila*, organ pipe cactus may be a nutritionally poorer substrate due to the triterpene glycosides.

With respect to viability and development time, our results agree very well with those of Etges and Heed (1987). As previously mentioned, they found that organ pipe tissue caused lower viabilities and longer development times than agria tissue for both mainland (Arizona) and Baja populations of *D. mojavensis*, especially at higher larval densities. Our data, however, strongly conflict with theirs on the subject of thorax sizes. They reported that, regardless of the larval density (e.g., 50, 100, 200, and 400 eggs/25 g cactus) and the population (mainland or Baja), thorax sizes of both males and females were smaller on organ pipe tissue than on agria. The results reported herein indicate that their observations on thorax sizes are not due to the cactus saponins since the saponins, by themselves, produce the opposite effect. A similar set of experiments using other chemical constituents of these cactus species would be useful in substantiating their data and implicating the chemical components (if any) that are responsible. Organ pipe lipids have been shown to significantly reduce the thorax size of *D. mojavensis* (Starmer, 1982), but, unfortunately, no comparable study using agria lipids has been done. Several chemical constituents of

the organ pipe lipid fraction (e.g., fatty acids and sterol diols) have been shown to have biological activity on fitness components of cactophilic *Drosophila* (Fogleman et al., 1986; Fogleman and Kircher, 1986), although they have not been individually tested for their effect on thorax size. Finally, it is possible that different geographic strains of *D. mojavensis* may vary in their response to cactus saponins. This idea, however, is not supported by the work of Etges and Heed (1987), since the two strains that they used (Arizona and the Baja Peninsula) were qualitatively identical with respect to their fitness response patterns.

In addition to their effect on fitness components, cactus triterpene glycosides have been implicated as playing a major role in host-plant selection by *D. mojavensis*. In general, *D. mojavensis* prefers agria over organ pipe in areas where the two cacti are sympatric (Fellows and Heed, 1972). Laboratory experiments have shown that this behavioral preference is determined by the pattern of volatile alcohols, esters, and fatty acids that are produced during microbial decay (Downing, 1985; Fogleman and Heed, 1989). The volatile pattern in naturally occurring necroses of agria and organ pipe are significantly different (Fogleman and Heed, 1989), and, since triterpene glycosides represent the major source of fermentable carbohydrates, they appear to be the chemical basis of host-plant selection, at least for *D. mojavensis*.

In summary, our results demonstrate that the saponins from agria and organ pipe cacti do affect the three fitness parameters that were measured for *D. mojavensis*. Triterpene glycosides from organ pipe increased development time and reduced larval viability while TTGs from agria produced smaller adults (reduced fecundity). In addition, the microbial communities in the organ pipe media were less dense than those in the agria media. It is difficult to state which effect(s) may be the most important to the flies with respect to inclusive fitness, but, if one views the behavioral preference for agria as a result of natural selection to maximize fitness, then necrotic organ pipe tissue is the poorer substrate. The triterpene glycosides appear to be involved in the ecology of this *Drosophila* species from the standpoints of both host-plant utilization and host-plant selection.

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Aplysia SEA HARE ASSIMILATION OF SECONDARY
METABOLITES FROM BROWN SEAWEED,
Styopodium zonale

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Abstract—Juvenile *Aplysia dactylomela* were found feeding in abundance on the tropical brown alga *Styopodium zonale*, a seaweed previously shown to contain numerous unique terpene-quinone natural products. Lipid extracts of these herbivorous mollusks were shown by TLC and HPLC-NMR analyses to contain appreciable quantities of two *S. zonale* metabolites as well as one new but closely related compound. Spectroscopic analyses of the new compound in concert with functional group modifications identified this new compound as 3-keto epitaondiol. A careful analysis of the seaweed extract failed to locate this ketone, and thus, it most likely represents an *Aplysia*-biotransformed compound. This is the first clear reported observation of metabolite transfer between an alga of the phylum Phaeophyta and a sea hare.

Key Words—Sea hare, *Aplysia dactylomela*, *Styopodium zonale*, mollusk, brown seaweed, Phaeophyta, diterpene-quinone, metabolite transfer, biotransformation, 3-ketoepitaondiol.

INTRODUCTION

There are numerous examples of the isolation of terpenoid compounds from sea hares of the genus *Aplysia*, which probably originate from their algal diet (Faulkner, 1984). Most of these examples are for the assimilation of red (Rhodophyta) algal secondary metabolites (Erickson, 1983). By contrast, there are relatively few metabolites reported from sea hares that have as an ultimate biogenetic origin a brown (Phaeophyta) (Amico et al., 1980; Gonzalez et al., 1983; Ireland et al., 1976; Midland et al., 1983; Sun and Fenical, 1979), green (Chlorophyta), or blue green algae (Cyanobacteria) (Rose et al., 1978). There are

only three circumstantial cases (isolation of structurally related metabolites from the two sources) in which it has been hypothesized that a sea hare assimilates the secondary metabolites of a brown alga. Specifically, related bicyclic diterpenes were isolated from the sea hares *Dolabella californica* (Ireland et al., 1976) and *Aplysia dactylomela* (Gonzalez et al., 1983) and from the brown algae *Dictyota dichotoma* (Amico et al., 1980) and *Glossophora galapagensis* (Sun and Fenical, 1979). Further, lactone-containing diterpenes related to acetoxycrenulide were isolated from both the sea hare *Aplysia vaccaria* and the brown alga *Dictyota crenulata* (Midland et al., 1983).

Thus, it was of interest to us to observe, in the context of a survey of the biomedicinal potential of Puerto Rican seaweeds (Ballantine et al., 1987), numerous juvenile *Aplysia dactylomela* feeding upon the tropical brown alga *Styopodium zonale*, a well-recognized source of several unique terpenoid secondary metabolites (Gerwick et al., 1979; Gerwick and Fenical, 1981). We were particularly curious to examine these herbivorous mollusks for the presence of any *S. zonale* natural products or related biotransformation products. The seaweed substances had been previously shown to be strongly toxic to predaceous tropical reef fish, and hence, of potential importance to the adaptive strategy of these plants (Gerwick and Fenical, 1981). By extension, it is possible that the assimilation of these brown algal metabolites by sea hares could be of importance to their survival in predator rich environments.

A combination of thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) spectroscopy were employed in the analysis of the lipid extract obtained from these mollusks and conclusively showed that several of the seaweed metabolites were present in appreciable quantities in the sea hares. Furthermore, a new, although closely related, compound was isolated from the sea hares and its structure determined by classic spectrochemical methods. Based on the structural similarities of this new compound to others reported from the seaweed, and found in the sea hares, this new compound most likely represents a biotransformation product. A careful examination of the seaweed blades upon which the *Aplysia* were found feeding failed to find detectable quantities of the new metabolite. It would appear that these sea hares are capable of assimilating the secondary metabolite chemistry from a wide variety of diets, including *S. zonale*, and furthermore that the secondary metabolites of a wide variety of algae can function in the sea hares' chemical defense strategy.

METHODS AND MATERIALS

General. Ultraviolet spectra were recorded on an Aminco DW-2a UV-Vis spectrophotometer and infrared spectra on a Perkin Elmer 727 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on Varian

EM 360, FT-80A and Bruker AM 400 spectrometers, and all shifts are reported relative to an internal TMS standard. Low-resolution mass spectra (LRMS) were obtained on a Varian MAT CH7 spectrometer, while high-resolution mass spectra (HRMS) were obtained on a Kratos MS 50TC. High-performance liquid chromatography (HPLC) was done using a Waters M-6000 pump, U6K injector, and R401 differential refractometer, while thin-layer chromatograms (TLC) were made using Merck aluminum-backed TLC sheets (silica gel 60 F₂₅₄). All solvents were distilled prior to use.

Collection and Extraction. A total of 133 individuals of juvenile (average fresh weight per animal = 130 mg, approximately 1 cm long) *Aplysia dactylomela* were collected from the surfaces of *Styopodium zonale* found growing in abundance in 2- to 6-m water depth at Vega Baja on the North Coast of Puerto Rico in June and July 1984. These were preserved in MeOH and subsequently repetitively extracted with chloroform and methanol (2:1). The chloroform-soluble material (956 mg) was analyzed by thin-layer chromatography and then preparatively fractionated employing vacuum chromatography over silica gel (Kieselgel). A steep gradient of isoctane-ethyl acetate-methanol was used to rapidly fractionate this extract into 13 fractions. Recombination of fractions 4-6 (eluting with 30-50% ethyl acetate in isoctane) gave 78.9 mg from which approximately 10 mg of (+)-epitaondiol (**2**) (identified from its high-field NMR spectrum and showing $[\alpha]_D = +31^\circ$; lit = $+43^\circ$) and 30 mg of the new compound (**3**) were isolated using HPLC (3.9 mm \times 50 cm μ -Porasil, 15% EtOAc-isoctane) (Scheme 1). *S. zonale* collected at the same time as the *Aplysia* collection was similarly stored in MeOH, extracted with CHCl₃-MeOH (2:1), and repetitively fractionated over silica gel in the vacuum mode to yield concentrated fractions of materials of the same polarity characteristics as the new compound.

3-Ketoepitaondiol (3). Pure **3** showed the following: IR (CHCl₃) 3380, 2950, 1700, 1605, 1480, 1380, 1145, 1130, 990, 860 cm⁻¹; LRMS (*m/z*, 70 eV) obs. M+ 410 (100), 382 (5), 273 (38), 217 (5), 215 (5), 205 (6), 203 (6), 189 (13), 175 (36), 161 (11), 137 (55), 121 (23), 107 (17), 95 (18), 81 (19), 58 (25), 55 (30); HRMS obs. M+ 410.2844 (C₂₇H₃₈O₃, 2.3 mamu dev., 1.5%); [¹H] NMR (400 MHz, CDCl₃) δ 6.45 (1H, d, *J* = 2.9 Hz), 6.39 (1H, d, *J* = 2.9 Hz), 4.31 (1H, bs), 2.72 (2H, m), 2.53 (2H, m), 2.31 (1H, ddd, *J* = 15.5, 12.2, 2.5 Hz), 2.09 (3H, s), 1.92 (2H, s), 1.74 (1H, dd, *J* = 13.4, 5.0 Hz), 1.28 (3H, s), 1.20 (3H, s), 1.09 (3H, s), 1.04 (3H, s), 0.81 (3H, s).

Acetate 4. The acetate was formed by stirring 10.1 mg of a crude fraction containing **3** in 1 ml pyridine and 1 ml acetic anhydride for 26.5 hr. The reaction was worked up by simple in vacuo removal of solvents to yield a mixture of related products. These were easily separated over HPLC (μ -Porasil, 3.9 mm \times 50 cm, 15% ETOAc in isoctane) to yield approximately 6 mg of **4**, which showed the following: $[\alpha]_D = +68.1^\circ$ (CHCl₃, *c* = 0.34); [¹H]NMR (400 MHz, CDCl₃) δ 6.64 (1H, bs) 6.62 (1H, bs), 2.75 (1H, dd, *J* = 13, 16 Hz),

are very limited; and (3) the assimilation and biotransformation of brown algal secondary metabolites by *Aplysia* is not well recognized. Hence, we made a substantial collection of these *S. zonale* grazing sea hares and have analyzed the crude lipid extract for the algal metabolites and related biotransformation products.

The alcohol-preserved specimens of *A. dactylomela* were extracted by normal methodology to yield a relatively rich organic extract, characteristic of the *Aplysia* sea hares (Faulkner, 1984). Comparison of this lipid extract with that obtained from the *S. zonale* plants upon which the sea hares were feeding showed that the distinctive red orthoquinone stypoldione (**1**), the major secondary metabolite in *S. zonale*, was clearly present in these animals (co-TLC with authentic material, approx. 5% of crude extract). Furthermore, by TLC analysis, several related compounds of a less polar nature were present in substantial quantities as well. Characterization of these, however, required vacuum liquid chromatography (Coll and Bowden, 1986) and HPLC separation followed by high-field NMR description.

Fractions eluting from a vacuum silica gel column with 30–50% ethyl acetate in isooctane were enriched in compounds which, on TLC, showed UV and acid-char characteristics similar to other known *S. zonale* secondary metabolites. These were combined and rechromatographed using normal-phase HPLC to give first the known compound (+)-epitaondiol (**2**) followed by a new compound (**3**), the spectroscopic structure elucidation of which is described below.

The new *Aplysia* natural product gave a small parent ion in the high-resolution mass spectrum, affording a molecular formula of $C_{27}H_{38}O_3$, a similar molecular formula to that of epitaondiol but containing an additional degree of unsaturation. The high-field [1H]NMR spectrum of **4** contained bands highly indicative of the diterpene-quinone structure type commonly observed in *Styopodium*. Specifically, two meta-coupled aromatic protons at δ 6.45 and 6.39 shifted to δ 6.64 and 6.62 in the monoacetate derivative (**4**). A broadened aromatic methyl group at δ 2.09 and a singlet methyl group at δ 1.28 in **3** provided further evidence for a chromane ring system as found in epitaondiol (**2**). A very favorable comparison between the chemical shifts and coupling constants for the coupling between H-2 β and H-1 α and H-1 β , the magnitudes of which are diagnostic for the stereochemistry of the protons at C-1 and C-2, showed the new compound to be stereochemically related to epitaondiol at C-2. Four additional methyl groups at chemical shifts similar to those found for epitaondiol were also in the high field [1H]NMR spectrum of **4**, suggesting a tricyclic skeleton as found in most of the other *Styopodium* metabolites. This was confirmed by high-field [^{13}C]NMR work, including a 1H - ^{13}C heteronuclear correlated NMR experiment, and provided a favorable comparison with epitaondiol [^{13}C]NMR shifts.

The major difference between the new metabolite (**3**) and other previously

described *Styopodium* compounds, such as epitaondiol (**2**), was the lack in the new compound of the C-14—CH(OH)— proton. Instead, the third oxygen in **3** was present as a ketone at C-14 as indicated by a 1700 cm^{-1} ketone stretch in the IR and a δ 219.9 carbonyl carbon in the ^{13}C NMR spectrum, thus accounting for the additional degree of unsaturation. The downfield chemical shift of this carbonyl is consistent with adjacent gem dimethyl groups, thereby further supporting the C-14 placement of this ketone functional group. Lack of sufficient sample precluded establishment of the absolute stereochemical relationship of 14-ketoepitaondiol (**3**) to naturally occurring (+)-epitaondiol (**2**). Most likely, however, this represents *S. zonale*-derived (+)-epitaondiol, which the sea hares have assimilated and selectively oxidized at the C-14 position.

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STRUCTURE-ACTIVITY STUDIES ON AGGREGATION
PHEROMONE COMPONENTS OF *Pityogenes*
chalcographus (COLEOPTERA: SCOLYTIDAE)
All Stereoisomers of Chalcogran and Methyl
2,4-Decadienoate

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Abstract—Syntheses of all four stereoisomers (2*S*,5*S*; 2*S*,5*R*; 2*R*,5*R*; and 2*R*,5*S*) of chalcogran, a major component of the aggregation pheromone of *Pityogenes chalcographus*, and of all four isomers (2*Z*,4*Z*; 2*Z*,4*E*; 2*E*,4*E*; and 2*E*,4*Z*) of methyl 2,4-decadienoate (MD), the second major pheromone component, are briefly described. Attraction responses of walking beetles of both sexes were tested to mixtures of the synergistic pheromone components or analogs. These bioassays showed that the *E,Z* isomer of MD is the most active when tested with chalcogran. When tested with (*E,Z*)-MD, (2*S*,5*R*)-chalcogran was the most active stereoisomer, while 2*R*,5*R* and 2*R*,5*S* isomers had intermediate activities, and the 2*S*,5*S* isomer was inactive. There was no evidence that the relatively less active stereoisomers of chalcogran inhibited or promoted attraction to (2*S*,5*R*)-chalcogran with (*E,Z*)-MD. Male beetles only produce the active *E,Z* isomer of MD (inactive alone) and their hindguts contain the most active (2*S*,5*R*)- and least active (2*S*,5*S*)-chalcogran. A mixture of all MD isomers with racemic chalcogran was not significantly different in attractivity compared to (*E,Z*)-MD with racemic chalcogran, indicating no synergistic or inhibitory effects of the inactive isomers of MD.

Key Words—Synergism, aggregation pheromone, *Pityogenes chalcographus*, Coleoptera, Scolytidae, chalcogran, methyl (2*E*,4*Z*)-2,4-decadienoate, enantiomers, isomers, stereoisomers, synthesis, bioassay, structure-activity.

INTRODUCTION

The six-spined spruce bark beetle, *Pityogenes chalcographus* (in Germany called *Kupferstecher*), is a serious pest in Europe of Norway spruce [*Picea abies* (L.) Karst.], especially the younger trees. Both sexes of these tiny beetles (2 mm long) aggregate on host trees in response to a pheromone (Vité, 1965). Francke et al. (1977) isolated from males a unique spiroketal, chalcogran, which when released in the forest at 15 mg/hr attracted conspecifics. They isolated chalcogran by treating 100,000 beetles of both sexes with a juvenile hormone analog to induce synthesis of chalcogran in males. They then used a differential diagnosis method (Vité and Renwick 1970) to compare the sexes for volatile chemical differences, which revealed *E* and *Z* isomers of chalcogran and 1-hexanol as being unique to the male. However, when 1-hexanol was added to synthetic racemic chalcogran (46% *E*:54% *Z*, Koppenhoefer et al., 1980), there was no apparent increase in attraction (Francke et al., 1977). Furthermore, due to the rather large amounts of chalcogran required to elicit attraction in the field (15 mg/hr), Francke et al. (1977) suggested that (1) "a second component may be necessary for maximum response" or (2) "it is also possible that the enantiomeric composition of the isomers is critical for maximum beetle response." Using gas chromatography (GC) on a chiral liquid phase, Koppenhoefer et al. (1980) were able to separate synthetic chalcogran containing all four stereoisomers into the individual components (two diastomeric pairs of enantiomers). Using the same technique, Schurig and Weber (1984) found that an extract of male beetles consists of a mixture of only two of the stereoisomers, namely the diastereomers 2*S*,5*R* 1 and 2*S*,5*S* 1 (Figure 1). However, the attraction activities of these, and the "unnatural" stereoisomers, have not been determined for male and female beetles.

Byers et al. (1988, 1989) further investigated the attractive pheromone system of *P. chalcographus* by undertaking an isolation of semiochemicals using chemical fractionation and behavioral bioassays. However, they used a new procedure, "subtractive combination" of gas chromatographic (GC) fractions and bioassay, which usually requires far fewer behavioral tests than the previously used and similar "additive combination" method (Silverstein et al., 1966, 1967, 1968; Pearce et al., 1975). Chalcogran and a "second" pheromone component were revealed by the new methods and found to be produced and released only when males feed on host-tree tissue. However, it was necessary to further isolate the second pheromone component from a complex mixture of insect and host volatiles by using two-dimensional GC (Deans, 1981) and bioassay (Byers et al., 1989). Methyl (2*E*,4*Z*)-2,4-decadienoate (*E*,*Z*-MD) was then isolated

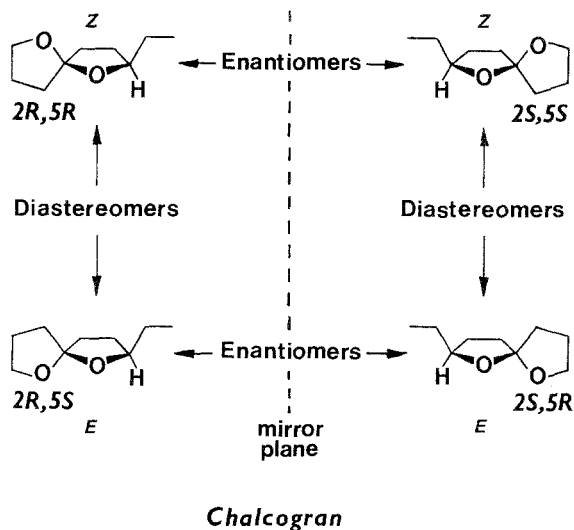


FIG. 1. Diastereomers and enantiomers of chalcogran. The two natural enantiomers found in male *Pityogenes chalcographus* are to the right of the mirror plane, with the behaviorally active one in the lower right.

and identified by GC-MS and found to be strongly synergistic with chalcogran (Byers et al., 1988, 1989). The males produce only the (*E,Z*)-MD isomer, but there are three other possible isomers that might have weaker or stronger synergistic effects on the attraction response when tested with chalcogran.

Here we briefly describe the syntheses of all four stereoisomers of chalcogran as well as the four isomers of methyl 2,4-decadienoate (MD) and their use in bioassay. The objectives were to determine which of the isomers/enantiomers of the pheromone components are bioactive by comparing (1) the attraction activities of each of the chalcogran stereoisomers when combined with the beetle-produced (*E,Z*)-MD and (2) the attraction activities of each of the MD isomers when combined with racemic chalcogran. We also wanted to determine if the mixture of chalcogran plus the four MD isomers has inhibitory or synergistic effects on attraction when compared to chalcogran plus the (*E,Z*)-MD isomer.

METHODS AND MATERIALS

Synthesis of Chalcogran Stereoisomers. Mixtures of the *E* and *Z* diastereomers of chalcogran were obtained enantiomerically pure using the synthetic pathway shown in Figure 2 (Högberg et al., 1987). The required γ -caprolactone enantiomers **2** (Figure 2) were conveniently obtained via resolution by chiral-

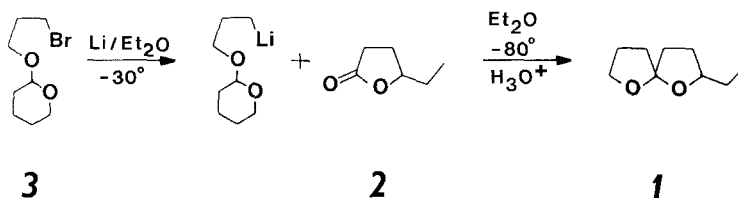


FIG. 2. Schematic pathway for synthesis of the chalcogran stereoisomers.

phase chromatography on microcrystalline triacetylated cellulose using the chromatographic system described by Isaksson and Roschester (1985). This gave (*R*)-(+)- and (*S*)-(–)- γ -caprolactone in 90% and 97% enantiomeric excess, respectively. These were subsequently reacted separately at -80°C with the alkyllithium obtained from the bromide **3** (Figure 2), each yielding a mixture of two diastereomers (*2R,5R/S*)-**1** and (*2S,5S/R*)-**1**, respectively. The diastereomers were separated by repeated silica gel chromatography using a gradient elution technique. All four isomers were obtained (Figure 1) in relatively high purity (Table 1) as shown by capillary GC on a chiral stationary phase (Schurig and Weber, 1984; Koppenhoefer et al., 1980).

Synthesis of Geometric Isomers of Methyl 2,4-Decadienoate. For details

TABLE 1. COMPOSITION OF CHALCOGRAN AND METHYL DECADIENOATE (MD) PHEROMONE COMPONENTS OR ANALOGS TESTED IN BIOASSAY

	Percent composition			
	<i>2S,5R</i>	<i>2R,5S</i>	<i>2S,5S</i>	<i>2R,5R</i>
(<i>E</i>)-Chalcogran				
<i>2S,5R</i>	96.2	1.7	2.1	<0.1
<i>2R,5S</i>	5.2	91.3	0.3	3.2
(<i>Z</i>)-Chalcogran				
<i>2S,5S</i>	1.0	<0.1	97.0	2.0
<i>2R,5R</i>	<0.1	0.9	3.8	95.3
Racemic chalcogran	23.0	23.0	27.0	27.0
	Percent composition			
MD	(<i>Z,Z</i>)	(<i>Z,E</i>)	(<i>E,E</i>)	(<i>E,Z</i>)
(<i>Z,Z</i>)	>99.9	<0.1	<0.1	<0.1
(<i>Z,E</i>)	<0.1	>99.9	<0.1	<0.1
(<i>E,E</i>)	<0.1	<0.1	99.0	<0.1
(<i>E,Z</i>)	<0.1	<0.1	<0.1	99.2

regarding the syntheses, see Baeckström et al. (1988). The *E,E* isomer was prepared (Figure 3) starting from methyl crotonate in a way similar to previously described procedures (Barneji and Pal, 1983; Crombie and Denman, 1984; Roush, 1980; Garigipati and Weinreb, 1983). NBS-bromination gave the product **4**, and an Arbuzov reaction with triethylphosphite yielded the phosphonate **5**. An Emmons-Horner-Wadsworth reaction with LDA and hexanal then gave (*E,E*)-MD. The isomeric purity was 93% and was improved to >99% using urea inclusion complexes (Leadbetter and Plimmer, 1979; Fieser, 1964). Since ethyl (*2E,4Z*)-2,4-decadienoate (**6**) is commercially available (Oril products Chimiques, Neuilly, France), the *E,Z* isomer was prepared simply by reesterification with sodium methoxide in methanol (Figure 3). The isomeric purity was increased from 85% to >99% with the urea inclusion procedure above. (*Z,E*)-MD was prepared from propiolic acid (Figure 3). HBr addition to the triple bond in the presence of cuprous bromide and subsequent esterification of the acid with sulfuric acid in methanol gave methyl (*Z*)-3-bromopropenoate (**7**) (Weir et al., 1980). This compound was coupled with (*E*)-1-heptenyl-1,3,2-benzodioxaborole (**8**) in the presence of Pd(PPh₃)₄ and sodium methoxide (Björkling et al., 1987). The cross-coupling reaction gave the *Z,E* isomer with 92% purity. Contaminating (*E,E*)-MD was removed by the use of urea inclusion

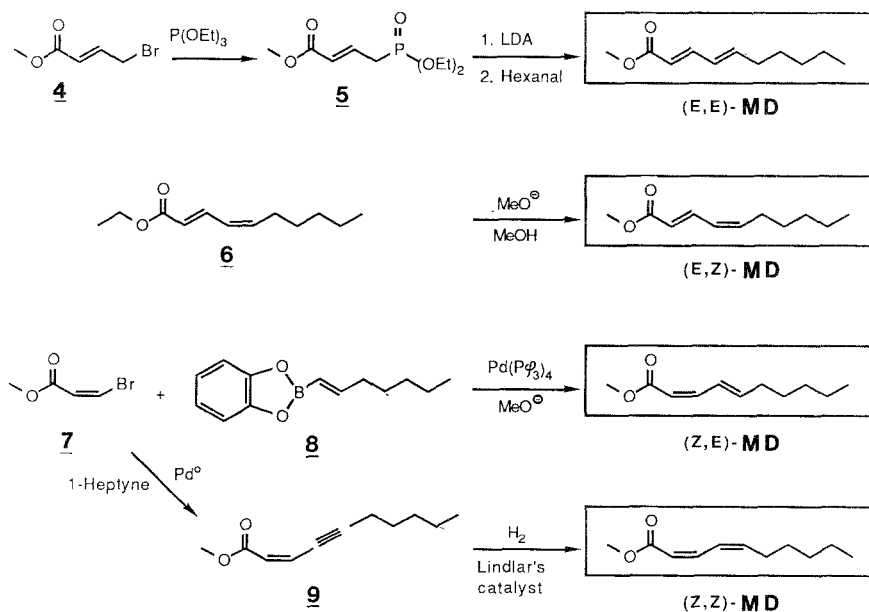


FIG. 3. Schematic pathway for synthesis of the geometrical isomers of methyl 2,4-decadienoate.

complexes. (*Z,Z*)-MD was prepared from **7** in two steps (Figure 3). A palladium-catalyzed cross-coupling with 1-heptyne according to Weir et al. (1980) yielded the enyne ester **9**. Reduction with hydrogen and Lindlar's catalyst (Rickards and Weiler, 1978) provided (*Z,Z*)-MD in 99% purity after preparative GC. The four MD isomers were analyzed and identified with GC-MS and [¹H]- and [¹³C]NMR, and the spectroscopic data were in agreement with those earlier reported (Stille and Groh, 1987).

Bioassay of Attraction Response of Pityogenes chalcographus to Chalcogran Stereoisomers and Methyl 2,4-Decadienoate Isomers. The open arena olfactometer for walking beetles was used for all bioassays (Browne et al, 1974; Byers and Wood, 1981; Byers et al., 1985). *P. chalcographus* (originally from Lardal, Norway) were obtained from a laboratory culture maintained on their natural host logs (28 × 10 cm diam.), Norway spruce, from Lund, Sweden. Beetles were separated according to sex and tested in groups of 10 in the olfactometer. They were released about 20 cm "downwind" from the odor source. Charcoal-filtered air was passed through a manifold to produce a laminar air flow across the arena of about 0.6 m/sec. Semiochemical mixtures were released in diethyl-ether solvent from a 5- μ l capillary (Drummond). Beetles that approached the odor source closer than 1 cm within the time of semiochemical release (about 2 min) were recorded as responding. Unresponsive beetles were tested a second time after refilling the capillary.

Purities of the chalcogran stereoisomers and MD isomers are shown in Table 1. Mixtures of each of the four chalcogran stereoisomers with the beetle-produced isomer of MD (Byers et al., 1988, 1989) were tested (Table 2) to see which stereoisomer was most active. Mixtures of racemic chalcogran (92.5% Shell Agrar, Table 1) with each of the four possible MD-isomers (Table 2) were also tested to determine their individual synergistic activities. Finally, tests were done to determine if the combination of all four MD-isomers were more active (synergism) or less active (inhibition) than the *E,Z* isomer when assayed with racemic chalcogran (Table 3).

RESULTS

The purities of the stereoisomers of chalcogran (Table 1) were sufficiently high to show differences in attraction activity (Table 2). In conjunction with (*E,Z*)-MD, (*2S,5S*)-chalcogran was the least active while the (*2S,5R*)-chalcogran was the most active for both sexes (Table 2). Again, in conjunction with (*E,Z*)-MD, racemic chalcogran [containing about 23% (*2S,5R*)-] was not significantly different from (*2S,5R*)-chalcogran in attraction activity, which indicates that the other stereoisomers of chalcogran are neither inhibitory nor synergistic (Table 2). The MD isomers were also of sufficiently high purity

TABLE 2. RESPONSES OF MALE AND FEMALE *Pityogenes chalcographus* IN LABORATORY BIOASSAY TO STEREOISOMERS OF CHALCOGRAN PRESENTED WITH (*E,Z*)-MD OR TO ISOMERS OF MD PRESENTED WITH RACEMIC CHALCOGRAN (JULY 27-28, 1986)^a

	Percent responding (95% BCL)	
	Females	Males
Chalcogran + (<i>E,Z</i>)-MD		
(2 <i>S</i> ,5 <i>R</i>) + (<i>E,Z</i>)-MD	55.0 (42.5-66.9)a	55.0 (42.5-66.9)a
(2 <i>R</i> ,5 <i>S</i>) + (<i>E,Z</i>)-MD	30.0 (19.9-42.5)b	23.3 (14.4-35.4)b
(2 <i>S</i> ,5 <i>S</i>) + (<i>E,Z</i>)-MD	5.0 (1.7-13.7)c	1.7 (0.3-8.9)c
(2 <i>R</i> ,5 <i>R</i>) + (<i>E,Z</i>)-MD	25.0 (15.8-37.2)b	23.3 (14.4-35.4)b
MD-isomers + chalcogran (<i>R</i>)		
(<i>Z,Z</i>)-MD + chalcogran (<i>R</i>)	25.0 (15.8-37.2)b	23.3 (14.4-35.4)b
(<i>Z,E</i>)-MD + chalcogran (<i>R</i>)	25.0 (15.8-37.2)b	25.0 (15.8-37.2)b
(<i>E,E</i>)-MD + chalcogran (<i>R</i>)	28.3 (18.5-40.8)b	25.0 (15.8-37.2)b
(<i>E,Z</i>)-MD + chalcogran (<i>R</i>)	60.0 (47.4-71.4)a	50.0 (37.7-62.3)a
Racemic chalcogran (<i>R</i>)	15.0 (8.1-26.1)b	11.7 (5.8-22.2)b
(<i>E,Z</i>)-MD	5.0 (1.7-13.7)c	5.0 (1.7-13.7)c

^aRacemic chalcogran (*R*) and each stereoisomer was released at 2.2×10^{-9} g/min and the MD isomers at 2.2×10^{-10} g/min. Parentheses enclose 95% binomial confidence limits for proportions (95% BCL) and 60 beetles of each sex were tested for each chemical blend. Percent responding followed by different letters were significantly different at $P < 0.01$, using a chi-square test.

TABLE 3. BIOASSAY FOR INHIBITORY OR SYNERGISTIC EFFECTS OF *E,E*, *Z,Z*, and *Z,E* ISOMERS OF METHYL DECADIENOATE (MD) ON RESPONSES OF MALE AND FEMALE *Pityogenes chalcographus* TO RACEMIC CHALCOGRAN PLUS (*E,Z*)-MD (OCTOBER 8, 1986)^a

	Percent responding (95% BCL)	
	Females	Males
Racemic chalcogran + (<i>E,Z</i>)-, (<i>E,E</i>)-, (<i>Z,E</i>)-, and (<i>Z,Z</i>)-MD	41.7 (30.1-54.3)	41.7 (30.1-54.3)
Racemic chalcogran + (<i>E,Z</i>)-MD	55.0 (42.5-66.9)	41.7 (30.1-54.3)

^aRacemic chalcogran was released at 2.2×10^{-9} g/min and each MD isomer at 2.2×10^{-10} g/min. Brackets enclose 95% binomial confidence limits for proportions (95% BCL) and 60 beetles of each sex were tested for each chemical blend. No significant differences were observed with a chi-square test, $P > 0.05$.

(>99%, Table 1) to yield differences in attraction activity (tested in combination with racemic chalcogran), with (*E,Z*)-MD being significantly more active than any other isomer or racemic chalcogran alone (Table 2). While (*Z,Z*)-, (*Z,E*)-, and (*E,E*)-MD tested with chalcogran were each somewhat more attractive than chalcogran alone, none of these combinations were statistically significantly different from chalcogran alone ($P > 0.05$, Table 2). The synergistically active (*E,Z*)-MD is not attractive when released alone (Table 2). When the mixture of all four isomers of MD plus chalcogran were compared to (*E,Z*)-MD plus chalcogran, there was no significant difference in attraction of either sex (Table 3), which indicates that the "inactive" MD isomers are neither synergistic nor inhibitory when combined with (*E,Z*)-MD.

DISCUSSION

Our bioassay tests revealed that the beetle-produced (*E,Z*)-MD was the only isomer of MD with significant synergistic activity with chalcogran. Apparently the configuration of both double bonds is important for the activity of the compound. The small activities of each of the other three MD isomers could be due to real effects or to the minute impurities of (*E,Z*)-MD. The expected release rates of any (*E,Z*)-MD impurity would have been on the order of 10^{-12} g/min or less, but this rate has produced a similar increase in response which is nearly or is statistically significant compared to response to chalcogran alone (Byers et al., 1989).

P. chalcographus males produce two of four possible stereoisomers of chalcogran (Shurig and Weber, 1984): (*2S,5R*)-1 and (*2S,5S*)-1, which we found were the most active and inactive, respectively, in our bioassay tests. There is some question as to the biosynthetic production of chalcogran because of rapid epimerization at carbon 5 to give a mixture of approximately 46% (*2S,5R*)-1 and 54% (*2S,5S*)-1 in solvents like benzene, ether, and chloroform (Smith et al., 1978; Francke et al., 1980). However, we found that our purified samples of chalcogran enantiomers were stable in hexane or pentane for over one year at -20°C by storage in alkaline-washed bottles. Thus, it is possible that the male beetle produces only one enantiomer that epimerizes in some part of the acidic gut to produce the 46:54 ratio as observed earlier (Francke et al., 1977; Shurig and Weber, 1984; Byers et al., 1989). Byers et al. (1989) found chalcogran predominantly in the abdomen (and more precisely in the hindgut; Birgersson and Byers unpublished) at 10.4 ± 2.4 ng (\pm SD) compared to only 2.6 ± 1.5 ng in the head/thorax. In contrast, (*E,Z*)-MD was found predominantly in the head/thorax of the male (11.2 ± 4.6 ng) compared to 2.3 ± 0.9 ng in the abdomen. This indicates different biosynthetic sites for the two pheromone components.

The structure-activities of the four chalcogran stereoisomers indicate that the configurations of all three major structural parts are important. Exchanging the ethyl and hydrogen groups at carbon 2 on the active (2*S*,5*R*)-chalcogran decreases response to an intermediate level for the (2*R*,5*R*)-**1** (Table 2). A 180° rotation of the center tetrahydrofuran ring on the active (2*S*,5*R*)-chalcogran also decreases response to an intermediate level for the (2*R*,5*S*)-**1**. Finally, 180° rotation of the end tetrahydrofuran ring at carbon 5 has the most severe effect, abolishing activity by forming (2*S*,5*S*)-chalcogran (Table 2).

Double-bond isomers of fatty acids of 12-, 14-, and 16-carbon chains are well known to have different behavioral effects or activities (Cardé and Baker, 1984). However, our report is the first case where such positional effects on long acetogenic carbon chains are important to bark beetles. It is well known that bark beetles use specific enantiomeric pheromone components (Wood et al., 1976; Borden et al., 1976; Wood, 1982), and *P. chalcographus* is yet another example. The *E* (2*S*,5*R*) and *Z* (2*S*,5*S*) chalcogran of *P. chalcographus* is analogous to *cis*- and *trans*-verbenol biosynthesis from α -pinene in *I. paracconfusus*, in which only one enantiomer of one compound is active, (–)-(*S*)-*cis*-verbenol (Silverstein et al., 1967; Renwick et al., 1976).

Our characterization of the synergistically bioactive stereoisomers of chalcogran (2*S*,5*R*)-**1**, and methyl 2,4-decadienoate, (*E*,*Z*)-MD, may be useful in control programs that use grids of pheromone-baited traps to catch the most vigorous (flying and responding) adults. This could reduce population levels below the tree-killing threshold so that tree resistance mechanisms can kill or repel the remaining potential colonizers. Pheromone trap-out may be a significant type of mortality which does not compete directly with other mortality factors such as adverse weather or desiccation/exhaustion of adults during the dispersal and host-seeking flight. The (*E*,*Z*)-MD and chalcogran components have recently been marketed under the name Chalcoprax, as a trap lure for *P. chalcographus* in Europe and northern Asia (Shell Agrar GmbH & Co. KG, D-6507 Ingelheim am Rhein).

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SENSITIVITY OF *Beauveria bassiana* TO SOLANINE AND
TOMATINE:
Plant Defensive Chemicals Inhibit an
Insect Pathogen

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Abstract—The alkaloids solanine and tomatine and the polyene antibiotic nystatin were tested *in vitro* against the entomopathogenic fungus *Beauveria bassiana*. Nystatin was the most inhibitory compound tested, reducing colony formation, growth, and development of conidiophores to a greater degree and at lower concentrations than the alkaloids. Tomatine inhibited colony formation and growth more than solanine, which had relatively little effect on the fungus. The toxicity of tomatine suggests that germination of conidia and subsequent hyphal growth would be inhibited when an insect consumes conidia along with foliage containing 0.100 mg/g (fresh weight) of this compound. The sensitivity of *B. bassiana* to alkaloids appears to be in the middle of the range found with other fungi.

Key Words—Entomopathogen, fungus, *Beauveria bassiana*, inhibition, plant defenses, alkaloids, solanine, tomatine, nystatin.

INTRODUCTION

Insect susceptibility to entomopathogens is influenced by the insect's host plant, yet the factors involved are poorly understood (Hare and Andreadis, 1983; Ramoska and Todd, 1985). Hare and Andreadis (1983) found that a host plant can indirectly influence the success of fungal infection with *Beauveria bassiana* through changes in insect fitness; less fit insects are more susceptible to infec-

tion. However, an anomaly in their results and the findings of Ramoska and Todd (1985) suggest that antifungal compounds in plants can have a direct, negative effect on *B. bassiana* and thereby retard infection of an insect. The glycoalkaloid tomatine was implicated in the reduced susceptibility to *B. bassiana* infection of the Colorado potato beetle, *Leptinotarsa decemlineata*, when fed tomato, *Lycopersicon esculentum* (Hare and Andreadis, 1983). The toxic and inhibitory effects of steroidal glycoalkaloids to fungal pathogens of plants has been reported often (Sinden et al., 1973; Arneson, 1967; Arneson and Durbin, 1967, 1968a,b; Allen, 1965; Wolters, 1964; McKee, 1959; Irving et al., 1946), but it is unknown whether glycoalkaloids are sufficiently toxic to *B. bassiana* to impede infection of an insect.

The toxicity of tomatine to fungi apparently results from the compound complexing with sterols in cell membranes and subsequently increasing membrane permeability (Arneson and Durbin, 1968a). Arneson and Durbin (1968a) mentioned that some fungi less sensitive to tomatine (Arneson, 1967) are also tolerant of nystatin, a polyene antibiotic with a similar mode of action (Fowlks et al., 1967; Kinsky, 1961). Tolerance in fungi can result from a lack of sterols in cell membranes for the alkaloids to bind to (Fowlks et al., 1967) or an ability to metabolize the compounds to a less toxic form (McKee, 1959; Arneson and Durbin, 1967). Determining the response of *B. bassiana* to nystatin helps to clarify its degree of tolerance to alkaloids in relation to other fungi and the general nature of the tolerance if found.

The undissociated bases of tomatine and the structurally similar alkaloids solanine and chaconine (Allen and Kuc, 1968) are probably the toxic form (Sinden et al., 1973; Arneson and Durbin, 1968a). While these alkaloids are all toxic and/or inhibitory to certain fungi, it can take approximately two and five times more solanine, depending on pH, than chaconine and tomatine, respectively, to have the same effect (Sinden et al., 1973; McKee, 1959). Because only unprotonated alkaloids complex with sterols, the inhibitory activity of tomatine is 300 times greater at pH 8.0 than at pH 3.0 (Arneson and Durbin, 1968a). Sodium and potassium also increase the toxicity of alkaloids to fungi (McKee, 1959), and when potassium is replaced by sodium in the buffer, the activity of nystatin is enhanced (Kinsky, 1961).

This investigation was undertaken to determine if alkaloids could suppress *Beauveria bassiana* as Hare and Andreadis (1983) suggested. Hare and Andreadis inoculated Colorado potato beetle larvae per os using a leaf disk, which put the *B. bassiana* and plant alkaloids in contact with each other inside the insect's gut. The gut pH of this insect is slightly acidic (Grayson, 1958), so a similar pH was used in our trials to allow for the effect of pH on alkaloid toxicity (McKee, 1959). Additionally, the response of *Beauveria* to alkaloids was evaluated in relation to that published for other fungi.

METHODS AND MATERIALS

The two glycoalkaloid treatments, solanine and tomatine, and the fungal antibiotic nystatin were incorporated into buffered and unbuffered oatmeal dodine agar (ODA) (Chase et al., 1986). The ODA consisted of either 1 liter deionized or 1 liter buffered water, and 20 g oatmeal, 20 g agar, 0.5 g dodine (65% AI), 0.01 g crystal violet, and 1 ml each of penicillin G and streptomycin sulfate stock solutions. The stock solutions contained either 0.7 g penicillin G or 1 g streptomycin sulfate in 10 ml sterile deionized water. The oatmeal was boiled for 20 min, vacuum filtered through cheese cloth, and the volume brought to 1 liter. The agar, dodine, and crystal violet were added before sterilization. The penicillin and streptomycin were added after cooling the media to 45°C.

Five concentrations (100, 40, 14, 5, and 1 mg/liter medium) of each alkaloid and nystatin (Sigma Chemical Co.) were tested. Stock solutions of the alkaloids were made by diluting each compound (4.3 mg/ml) with 10 mM HCl. An appropriate amount of the stock solution to give the desired concentration of the test compound, along with enough 10 mM HCl to make the volume 6 ml, was added to 250 ml of ODA before plating; controls received only the 10 mM HCl. The pH in the buffered ODA was set at 6.35 with a buffer (500 ml of 0.1 M KH_2PO_4 + 126 ml of 0.1 M NaOH brought to 1 liter with distilled water), whereas the pH in unbuffered ODA was adjusted to 6.35 with 0.1 M NaOH after addition of the treatment chemicals. A set of 12 plates for each treatment concentration and 36 plates for controls were made from randomly selected batches of ODA.

Beauveria bassiana conidia (technical powder; Abbott Labs, ABG-6178 lot No. 76015BR) were diluted serially with deionized water containing 0.05% Tween-80 (pH 6.35). Each plate was inoculated by spreading 0.5 ml of the conidia preparation on the agar with a bent glass rod. Inoculated plates were wrapped in plastic to conserve moisture and incubated in the dark at 25°C. The number of colonies present was recorded four days after plating, and colony diameter was measured five and seven days after plating. Measurements of the two colonies first appearing in the microscopic field that were not crowded by other colonies were taken from each plate. The magnification used was varied with colony size. Colonies were examined for the presence of conidiophores on day 7.

An analysis of variance was used to determine the effects of media, treatment compound, and concentration on number of colonies, and their diameter during the first and second measurement. The colony counts were transformed before analysis by taking the base 10 log of each count. Tukey's multiple-range test was used to determine significant differences among treatments and dosages. A probability level of 0.05 was used in all statistical analyses.

RESULTS

Nystatin restricted colony formation to a greater extent and at lower concentrations than either alkaloid treatment (Figure 1a and b). The fungal response to alkaloids varied and was influenced by buffering agents. Both alkaloids inhibited colony formation on unbuffered media except for solanine at 1 and 40 mg/liter (Figure 1b). However, when the media were buffered, the alkaloids stimulated colony formation at lower concentrations, and in greater amounts had no significant effect (Figure 1a).

The measurements of colony diameter five days after plating revealed that both alkaloids had reduced colony size compared to the control, with tomatine having the greatest effect (Figure 2a and b). The response to solanine was most apparent at 5 mg/liter, the next-to-lowest concentration tested. This concentra-

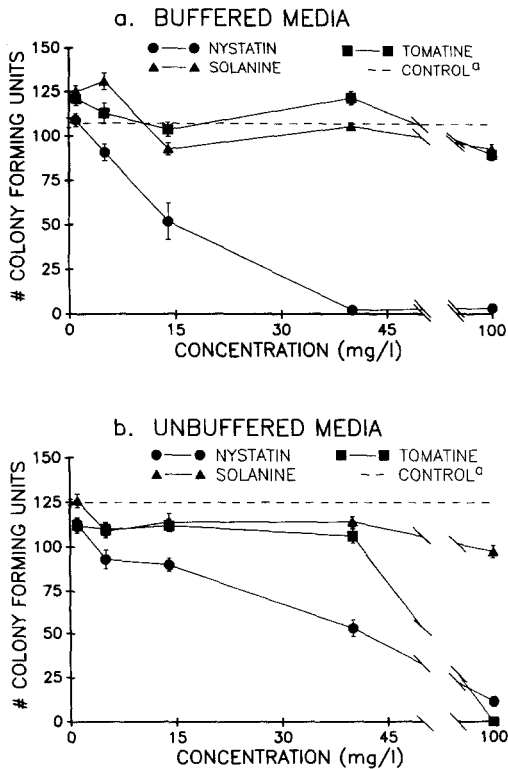


FIG. 1. Colony formation by *Beauveria bassiana*. Vertical lines indicate + or - one standard error and those symbols without lines had standard errors \leq the size of the symbol. ^aOnly tested at 0 mg/liter but line is extended for purpose of comparison.

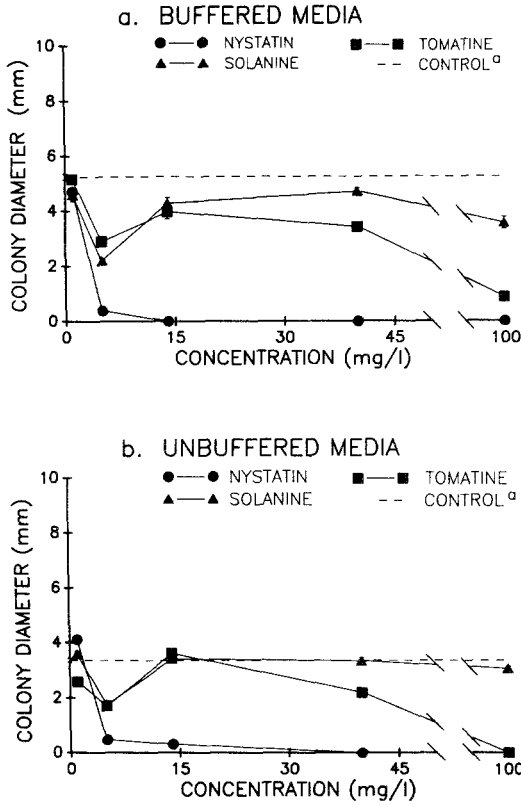


FIG. 2. Colony diameter five days after plating. Vertical lines indicate + or - one standard error and those symbols without lines had standard errors \leq the size of the symbol. ^aOnly tested at 0 mg/liter but line is extended for purpose of comparison.

tion of tomatine inhibited growth more than the next higher concentration tested (14 mg/liter) and in unbuffered media 1 mg/liter also had a negative effect. However, alkaloids incorporated into buffered media were generally more inhibitory to fungal growth than when the medium was not buffered. The complete inhibition of colony formation by the highest dose of tomatine in unbuffered medium was the exception. Little or no growth was observed on plates containing nystatin in all but the lowest concentration tested.

Colonies on plates containing nystatin had increased in diameter by the seventh day after plating, but remained significantly smaller than those on most alkaloid treatments (Figures 3a and b). Several concentrations of tomatine continued to inhibit colony growth in both media, whereas solanine's influence was most apparent when the media were buffered. The initial retardation of growth

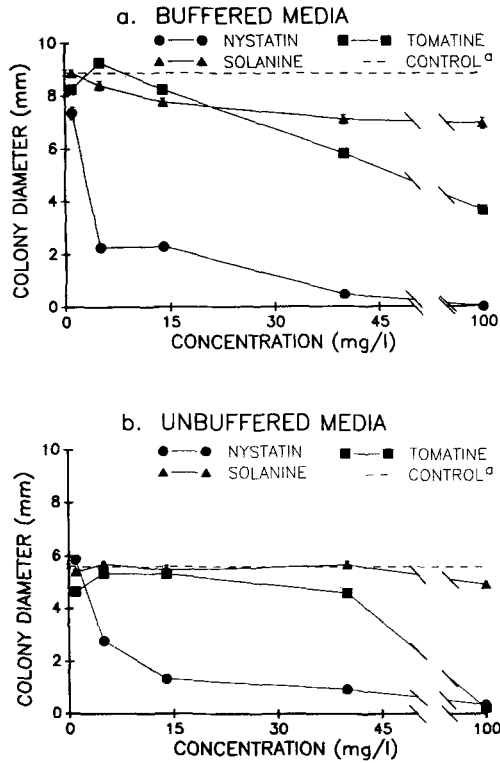


FIG. 3. Colony diameter seven days after plating. Vertical lines indicate + or - one standard error and those symbols without lines had standard errors \leq the size of the symbol. ^aOnly tested at 0 mg/liter but line is extended for purpose of comparison.

caused by 5 mg/liter of the alkaloids (Figures 2a and b) had diminished by this time, but in unbuffered medium, 1 mg/liter of tomatine was still inhibitory, while the two successively higher concentrations were not.

The presence of conidiophores in a particular treatment was an all-or-nothing response, either all colonies observed had them or none did. Conidiophores formed at all the concentrations of solanine tested and all but the highest concentration of tomatine in unbuffered medium (Table 1). Only the lowest concentration of nystatin permitted production of conidiophores in either medium. This datum was not treated statistically because of the absolute nature of the response.

The presence or absence of buffer in the media had a significant influence on colony size; this is evident when the controls from both media are compared (Figures 2 and 3). The type of medium also interacted significantly with treat-

TABLE 1. PRODUCTION OF CONIDIAPHORES BY *Beauveria bassiana* WHEN EXPOSED TO SOLANINE, TOMATINE, AND NYSTATIN IN BUFFERED AND UNBUFFERED MEDIA

Concentration (mg/liter)	Buffered			Unbuffered		
	SOL ^a	TOM	NYS	SOL	TOM	NYS
0	+ ^b	+	+	+	+	+
1	+	+	+	+	+	+
5	+	+	0	+	+	0
14	+	+	0	+	+	0
40	+	+	0	+	+	0
100	+	+	0	+	0	0

^aSOL = solanine; TOM = tomatine; NYS = nystatin.

^b+ = produced conidiaphores; 0 = did not produce conidiaphores.

ment compound and concentration for all the parameters measured, except conidiaphore production, which was not analyzed statistically.

DISCUSSION

The negative response of *B. bassiana* to tomatine was greater than with solanine, but was it enough to substantiate the suggestion of Hare and Andreadis (1983) that tomatine might inhibit infection of an insect? Although the tomatine content of the tomato variety Rutgers that Hare and Andreadis (1983) used is not available to us, Campbell and Duffey (1981) presented data for five other varieties. The unweighted mean from their results for all the varieties was 0.163 mg/g (fresh weight) or an equivalent of 163 mg/liter in agar medium. Colony formation and growth of *B. bassiana* were severely inhibited by 100 mg/liter of tomatine in unbuffered media, implying that higher concentrations would also be inhibitory. Therefore, the tomatine presumably present in the Rutgers variety could have had a toxic effect on the fungus and retarded insect infection.

Tomatine's greater ability to inhibit *B. bassiana*, compared to that of solanine, parallels the findings of McKee (1959) with nonentomopathogenic fungi. Solanine's limited ability to retard fungal growth tends to support Hare and Andreadis's (1983) conclusion that the decreased susceptibility of insects fed *S. tuberosum* was a result of their greater fitness. Although the solanine and chaconine content previously reported for the variety they used was not tested in this trial (Gregory et al., 1981; Raman et al., 1979), given the trends observed, an influence far less than that caused by tomatine would be expected.

The smaller colonies and slower growth in the unbuffered control indicate

poor nutrition of the fungus; this may, in turn, have contributed to tomatine's greater toxicity at its highest concentration in this medium. Sodium could have been a limiting nutrient. No more than 0.2 mM Na was added when initially adjusting the pH, whereas 12 mM was incorporated into the buffered media—concentrations above 86 mM do not increase the growth response of *B. bassiana* (Samsinakova, 1966). The greater toxicity might also have resulted from a rise in medium pH, which occurs with *B. bassiana* growth (Samsinakova, 1966). McKee (1959) reported that the growth of *Fusarium caeruleum* increased media pH and resulted in eventual toxicity of solanine. However, the pH increase was larger than what Samsinakova (1966) reported for *B. bassiana*.

The greater toxicity of tomatine at its highest concentration in unbuffered media does not coincide with the tendency toward an increased alkaloid toxicity in buffered media. The additional sodium and potassium in the media could have increased the influence of the compounds as they did with solanine's toxicity to *F. caeruleum* (McKee, 1959). However, this effect did not extend to the initial colony formation after plating. Lower concentrations of the alkaloids stimulated the formation of colonies in buffered media.

The sensitivity of *B. bassiana* to alkaloids appears to be in the middle of the range reported for other fungi. The corn pathogen *Drechslera* (= *Helminthosporium*) *carbonum* is more sensitive than *B. bassiana*, having a 50% reduction in growth caused by 61 mg/liter of solanine at a lower pH (Allen and Kuc, 1968), which would have made the alkaloid less toxic. The potato pathogen *Alternaria solani* is similar in sensitivity, having only an 8% reduction in growth by 100 mg/liter (Sinden et al., 1973); *B. bassiana* growth was reduced 23% by the same amount at a comparable pH. The conidia of *B. bassiana* are similar in their sensitivity to tomatine to those of *F. caeruleum*, a potato pathogen that can hydrolyze solanine (McKee, 1959).

The negative response of *B. bassiana* to nystatin, although greater than with alkaloids, also indicated a tolerance relative to other fungi. Colonies developed, albeit poorly, with concentrations of nystatin up to 40 mg/liter and produced conidiophores with 1 mg/liter present, whereas *Colletotrichum lagenarium* and *Neurospora crassa* are completely inhibited by 0.6 and 2 mg/liter (Fowlks et al., 1967; Kinsky, 1961). The tolerant fungus *Pythium aphanidermatum*, which apparently has no sterols in its cell membrane for binding nystatin, was not inhibited by 100 mg/liter of the antibiotic (Fowlks et al., 1967). The response of *B. bassiana* to nystatin and its concurrent sensitivity to alkaloids suggest that *Beauveria*'s cell membranes possess sterols.

The relative tolerance of *B. bassiana* to alkaloids and nystatin implies that some mechanism may have evolved to deal with these compounds. The ability of certain fungi to hydrolyze alkaloids to less toxic forms has been demonstrated in plant pathogens (Arneson and Durbin, 1967; McKee, 1959). In our trial, colony size was initially smaller in the 5-mg/liter alkaloid treatments than in

the next higher concentration, and subsequently the effect diminished. This suggests that the tolerance was due to a detoxification system that is activated only when sufficiently challenged, rather than to a lack of sterols for binding the compounds. The tolerance of fungi specializing on alkaloid-containing plants is understandable given the degree of selective pressure they encounter. Less clear is how this pressure could be strong enough on an entomopathogenic fungus.

Further research is required to fully explain the response of *B. bassiana* to alkaloids. The relative tolerance of *B. bassiana* to alkaloids compared to other fungi needs to be confirmed with assays that test sensitivities of several fungi concurrently. A potential complication is that local populations of the fungus may vary in alkaloid tolerance as they do in their tolerance of pesticides (Anderson and Roberts, 1983; Olmert and Kenneth, 1974). Finally, consideration of *Beauveria's* response to alkaloids and other phytochemicals (Ramoska and Todd, 1985) should be incorporated into the development of strategies seeking to use this pathogen for the control of insect pests.

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PHEROMONE EMISSION BY INDIVIDUAL FEMALES OF CARNATION TORTRIX, *Cacoecimorpha pronubana*

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Abstract—The emission of five pheromone components by individual *Cacoecimorpha pronubana* females was measured during 15-min sampling intervals. Pheromone was only released during female calling. Blend ratios emitted by individual females were constant over three calling periods, despite variations in release rates. Release ratios of the $\Delta 11$ -components Z11-14:Ac, E11-14:Ac, and Z11-14:OH were similar for all females tested, but release ratios of Z9-14:Ac to the $\Delta 11$ -components showed significant interindividual variation.

Key Words—*Cacoecimorpha pronubana* Hbn., Lepidoptera, Tortricidae, sex pheromone emission, release rates, blend ratios, calling behavior, airborne collection.

INTRODUCTION

Airborne pheromones emitted by individual calling female moths have been trapped on substrates like Porapak Q and Tenax, on glass-wool or active carbon (Golub and Weatherston, 1984). Such adsorbants possess large surfaces with respect to the quantity of emitted pheromone. The subsequent elution of adsorbed compounds from these materials may result in contamination and loss of pheromone. Analysis of pheromone emission has thus often been confined to the measurement of only the main component during sampling intervals of 30 min and more.

Baker et al. (1980) demonstrated the use of glass surfaces for the efficient

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adsorption of pheromone without introducing impurities from the adsorbant. Trapping pheromones in glass capillary tubes allows desorption with minimal quantities of solvent for direct sample analysis (Shani and Lacey, 1984). In collection systems designed by Sower et al. (1971) and Baker et al. (1981), pheromone was collected from forcibly extruded female glands. This technique has been adapted by several authors to isolate minor pheromone components (Pope et al., 1982, Haynes et al., 1984, Du et al., 1987). Other methods have been developed where females were allowed to assume the natural calling position during collection (Bjostad et al., 1980, Charlton and Cardé, 1982, Shani and Lacey, 1984, Schal and Cardé, 1985).

The carnation tortrix, *Cacoecimorpha pronubana* is an important, multi-voltine pest of various flower, vegetable, and fruit cultures (Bovey, 1972). The sex gland secretion of *C. pronubana* contains approximately 80% (*Z*)-11-tetradecenyl acetate (*Z*11-14:Ac), together with a number of related alcohols and acetates, including (*E*)-11-tetradecenyl acetate (*E*11-14:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac), tetradecyl acetate (14:Ac), and (*Z*)-11-tetradecen-1-ol (*Z*11-14:OH) (Descoins et al., 1984, Frérot et al., 1989). A detailed study of the release rate of pheromone by individual females was undertaken as a prerequisite to the analysis of male pheromonal behavior.

METHODS AND MATERIALS

Insects. Insects were obtained from a continuous culture maintained in the laboratory (for approx. 30 generations) on a semiartificial diet. The laboratory strain originating from Antibes (southern France) in 1983 was regularly interbred with wild insects collected at the same site. Sexed pupae were kept under a 14:10 light-dark photoperiod at 80% relative humidity. After adult emergence females were kept individually in 200-ml plastic jars and fed with a 2% sucrose solution when not being tested.

Collection Apparatus. Pheromone collection was performed with an apparatus (Witzgall, 1987) integrating important features of the collection systems described by Shani and Lacey (1984) and Charlton and Cardé (1982). A glass bulb divided in two, fitted with a ground glass joint, contained the insect. The calling female rested freely on a metal support held by a wire fixed to the upper half of the bulb. A capillary tube (1 mm ID, 15 cm long) was connected to the outlet of the bulb. The extruded gland was next to the orifice (Figure 1C). A constant flow (40 cm³/min) of purified air (activated carbon) passed over the female into the capillary tube. The lower part of the glass cage could be rapidly exchanged without disturbing the female.

Preliminary Measurements with Synthetic Pheromone and Calling Females. To evaluate the efficiency of pheromone collection in this apparatus,

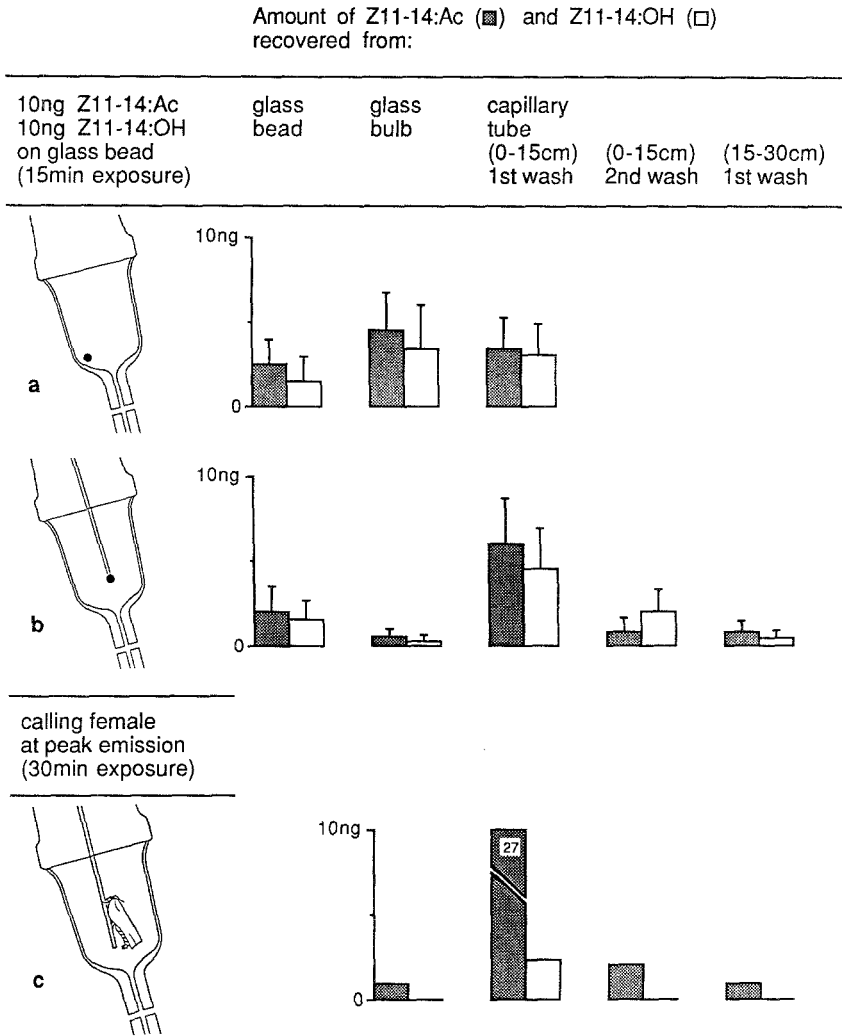


FIG. 1. Amount of the pheromone components Z11-14:Ac and Z11-14:OH recovered by elution with hexane from different parts of collection device. a, b: synthetic pheromone applied to a glass bead; $N = 10$. c: calling *C. pronubana* female.

we first undertook measurements with synthetic pheromone components. A 5- μ l hexane solution containing 10 ng of Z11-14:Ac and 10 ng of Z11-14:OH was applied to the round tip of a glass rod. After solvent evaporation, the dispenser was either fixed to the metal support or placed on the surface of the glass bulb with the tip 1 cm from the outlet (Figure 1A and B). One to two minutes

elapsed between application of the pheromone solution and the onset of the air flow. The glass dispenser was washed with 30 μ l hexane (Merck, for residue analysis) 2 min after solvent application and after a 15-min exposure in the collection apparatus. The lower part of the holding chamber was washed with 20 μ l hexane before and after application of the synthetic source. The first half, near the source (0–15 cm) of a capillary tube (30 cm long; broken in two), was rinsed with 5 μ l of hexane twice and the second half (15–30 cm) once. Amounts of Z11–14:Ac and Z11–14:OH recovered were quantified by GC analysis.

The recovery of 10 ng synthetic Z11–14:Ac and Z11–14:OH emanating from the artificial dispenser in different parts of the collection system after a 15 min exposure is shown in Figure 1A and B. The adsorption of synthetic pheromone in the lower part of the bulb was 4.7 ± 2.2 ng Z11–14:Ac ($N = 10$), when the artificial dispenser was in contact with the glass cage. With the dispenser fixed to a metal support in the center of the glass bulb, the total amount of pheromone detected in the bulb never exceeded 1 ng (10%) and the amount recovered from the capillary tube increased accordingly. This result and the observation that calling female moths usually remain in the same position led to the design of our glass-collection system shown in Figure 1C.

Recovery efficiency of the first wash of the capillary tube was generally lower for Z11–14:OH than for the corresponding acetate, although more Z11–14:OH was detected by the second wash. After direct injection of 10 ng Z11–14:Ac and 10 ng Z11–14:OH (5 μ l hexane solution) into the tube, followed by elution of the capillary after solvent evaporation, we recovered both compounds in an 85:74 ratio (8.5 ± 0.7 ng, 7.4 ± 0.6 ng, $N = 10$). Extraction of the dispenser 2 and 15 min after solvent application revealed similar recovery rates. This suggests a stronger adsorption of Z11–14:OH to the glass surface. Cooling of the tubes did not increase recovery rates of either compound. Silanization of the capillaries slightly increased the amount of Z11–14:OH recovered but was not applied routinely.

When females were placed directly in the glass bulb, the amount of Z11–14:Ac (the main pheromone component) measured in the capillary never exceeded 3 ng for 15-min intervals. Even 1 hr after the calling female was removed from the bulb, pheromone could still be detected in the capillary. This was probably due to secondary desorption of pheromone from the glass surface of the bulb. The minor components Z9–14:Ac, E11–14:Ac, 14:Ac and Z11–14:OH were only to be detected during peak emission. Up to 24.5 ng of Z11–14:Ac was detected for 15-min intervals in the capillary, when the female was placed on the metal support. The amount of pheromone eluted from the bulb was then always less than 5% of the amount detected in the capillary (Figure 1C).

Measurement of Female Pheromone Emission. *C. pronubana* is day-active. Insects emerge during the dark period and start calling during the following

light period. Ten females were tested individually zero, two, and four days after emergence during the entire calling period. Females were allowed to climb the support (Figure 1C) 1 to 2 hours after onset of the light period. They started calling 30–60 min later and rested on the platform during calling and when capillary tubes and the lower part of the holding chamber were exchanged. Calling behavior was observed at 15-min intervals. Pheromone was collected twice an hour during 15-min periods (0–15 and 30–45 min) into a capillary tube 15 cm long. Tube eluates were immediately submitted to GC analysis. The first rinse of the capillaries with 5 μ l hexane containing 5 ng of each 12:OH and Z9, E12–14:Ac as internal standards was used to quantify pheromone emission according to peak areas. A single factor analysis of variance showed that the amounts of 12:OH and Z9, E12–14:Ac did not differ among measurements with different females. All data represent actual values and were not adjusted. Tubes were used only once. The lower part of the glass cage was exchanged once an hour.

For statistical analysis of release ratios, we calculated the integrals of the emission rate of each compound for each calling period, since the consecutive measurements during 15-min intervals could not be treated as independent variables. To test for differences in the release ratios of any two compounds between females, we performed a Kruskal-Wallis test with tied ranks (Weber, 1980) on the integrals of the release rates.

GC Analysis. Hexane rinses were injected without further treatment on a Girdel 300 with Ross injector and FID detector (250°C) equipped with a fused silica column (WCot, CPWax 57 CB, 25 m, 0.22 mm ID). Helium was used as carrier gas, column temperature was held at 150°C.

RESULTS

Release Rates and Release Patterns. The 10 females tested on the first, third, and fifth days released an average of 0.45 ± 0.11 ng Z11–14:Ac/min/calling period, as measured in a 15-cm capillary tube. A similar pattern of release was observed for all five chemical components: Release rates were highest after the onset of calling, when the female lifted the abdomen from the platform and extruded the gland. Generally, release rates declined towards the end of the calling period and ceased when females stopped calling. No pheromone was detected before and after the calling period.

A typical diel pattern of pheromone release by an individual female is shown in Figure 2. It is important to note that pheromone release was correlated with the extrusion of the gland. Continuous observation of the calling behavior showed that after peak emission, females sometimes slightly retracted the gland for periods of 30 to 60 min and lowered the abdomen. Such partial retraction

of the gland generally resulted in important decreases of pheromone emission (Figure 2). However, emission never ceased entirely between the onset and the end of the calling period.

Release Ratios. The average composition of the bouquet Z11-14:Ac, E11-14:Ac, Z9-14:Ac, 14:Ac, Z11-14:OH released by the 10 females was 82:5:1:5:7. Despite the temporal variations in overall release rates, release ratios between the components detected were surprisingly constant for individual females. A given female emitted, for instance, Z11-14:Ac/Z11-14:OH, or Z9/E11-14:Ac at almost the same ratio during the entire calling period on the three days tested (Figure 3A and B).

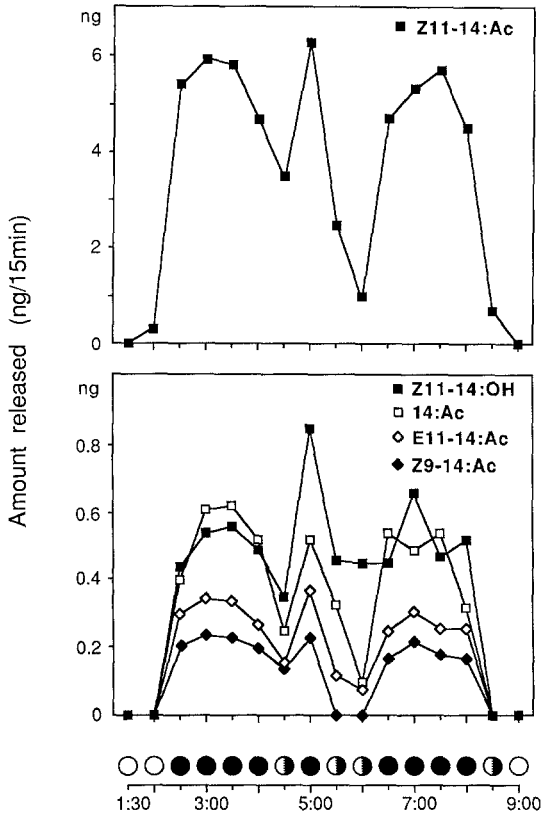


FIG. 2. Pheromone release by an individual *C. pronubana* female at two 15-min sampling intervals per hour, over an entire calling period. Circles at bottom denote calling behavior at the beginning of each sampling interval. Solid circle: fully extruded pheromone gland, abdomen lifted high from platform. Half circle: gland less extruded, lowered abdomen. Open circle: gland not visible, abdomen on platform. The female was placed on the support 1.5 hr after onset of the light period.

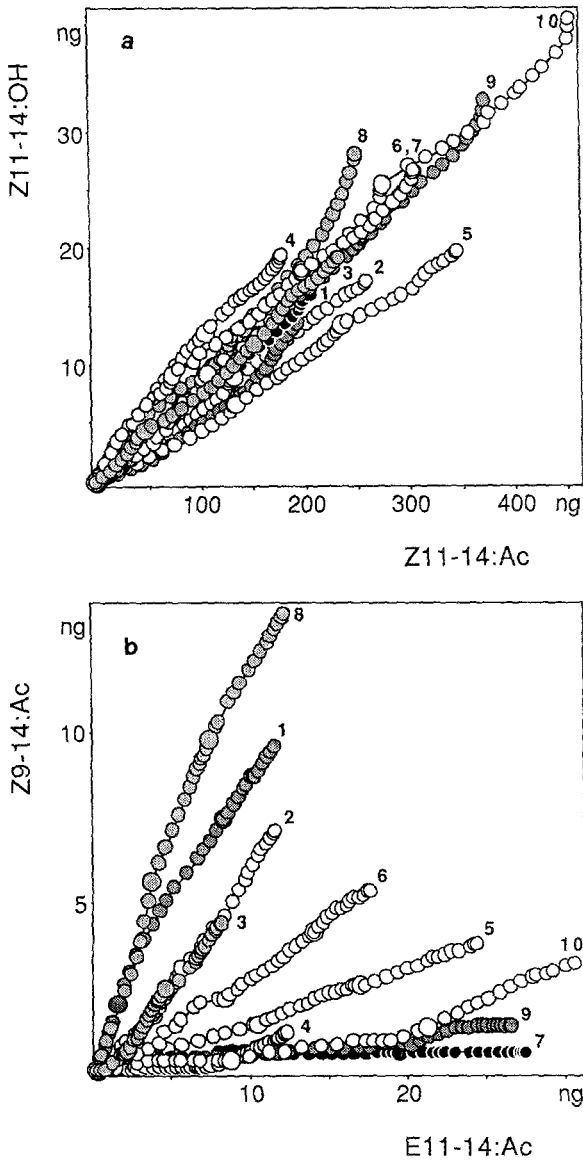


FIG. 3. Cumulative amounts of Z11-14:Ac/Z11-14:OH (a) and E11-14:Ac/Z9-14:Ac (b) released by 10 individual *C. pronubana* females over three calling periods 0, 2, and 4 days after emergence. Larger circles denote the first measurement of each calling period.

This individual constancy of release ratios is contrasted by the strong inter-individual variation of release ratios between Z9-14:Ac and the Δ 11-components. For example, the Z9-14:Ac/E11-14:Ac release ratio (Figure 3B), ranged from 0.04 to 1.17. A nonparametric analysis of variance (Kruskal-Wallis test; $P = 0.05$) showed that not only the Z9/E11 ratio, but also Z9/Z11-14:Ac and Z9-14:Ac/Z11-14:OH ratios were significantly different between females.

On the other hand, no significant interindividual variation could be detected for the release ratios between the Δ 11-compounds Z11-14:Ac, E11-14:Ac, Z11-14:OH. For example, the Z11-14:Ac/Z11-14:OH ratio measured was almost the same for all 10 females and ranged from 0.06 to 0.11 (Figure 3A). In this case, the actual emission ratio should be slightly higher, as less alcohol was eluted from the glass surface by comparison with the corresponding acetate (Figure 1A and B). Similarly, the 14:Ac/Z9-14:Ac ratio did not vary significantly between females.

DISCUSSION

The glass-adsorption collection device described in this study proved efficient for short-term measurement of five components of the sex gland secretion emitted by individual *Cacoecimorpha pronubana* females. A blend of four of these compounds (Z11-14:Ac, E11-14:Ac, Z9-14:Ac, and Z11-14:OH) has been shown to elicit male pheromonal behavior in the wind tunnel and in the field as observed with calling female moths (Witzgall, 1989).

A quantitative assessment of the minor pheromone components during 15-min intervals even outside peak emission periods was possible only after a modification of the collection system described by Shani and Lacey (1984). When the female was placed on a support within a glass bulb (as in Bjostad et al., 1980) the amount of pheromone trapped in the glass capillary tube increased appreciably. Pheromone is otherwise adsorbed within the glass bulb (Figure 1A and B), or probably on the body of the female (Baker et al., 1980; Ramaswamy and Cardé, 1984). In addition, secondary desorption of pheromone from the bulb modifies subsequent measurements.

In our system, pheromone could only be detected when females assumed the typical calling position (Figure 1C). A similar correlation between female calling and pheromone release has been established in *Trichoplusia ni* (Bjostad et al., 1980), *Choristoneura fumiferana* (Ramaswamy and Cardé, 1984), *Ephestia cautella* (Barrer et al., 1987), and *Holomelina lamae* (Schal et al., 1987). Interestingly, pheromone emission from forcibly extruded glands of *Heliothis virescens* (Pope et al., 1982) or *Platyptilia carduidactyla* (Haynes et

al., 1983) showed a diel periodicity similar to the calling cycle of undisturbed females. It therefore appears that gland extrusion merely coincides with the availability of pheromone for release. Gland extracts of individual *C. pronubana* females contained up to 100 ng Z11-14:Ac at the onset, but only a few nanograms at the end of the calling period (Frérot and Witzgall, 1989).

Blend proportions were remarkably constant for each individual female during three calling periods (Figure 3). This is consistent with results on *Ephesia cautella* (Barrer et al., 1987) and *Yponomeuta padellus* females (Du et al., 1987). In addition to this notable individual constancy of blend composition, we found no significant interindividual variation for the release ratios between the $\Delta 11$ -components [E11/Z11-14:Ac, Z11-14:Ac/OH (Figure 3A), E11-14:Ac/Z11-14:OH]. Rigid control of the E/Z11-14:Ac blend ratio in *Argyrotaenia velutinana* (Miller and Roelofs, 1980) has been shown to be the result of a common biosynthetic pathway of these two compounds (Bjostad and Roelofs, 1981). As functional groups of pheromone components can be readily interconverted in the insect (Morse and Meighen, 1986; Teal and Tumlinson, 1986, 1987; Wolf and Roelofs, 1987), we suspect that biosynthesis of $\Delta 11$ -14 alcohols and acetates follows the same pathway.

Most conspicuous, however, was the broad interindividual variation of the Z9/ $\Delta 11$ ratios (for example Z9/E11-14:Ac, Figure 3B). This considerable range in release ratios corresponds well with the results of our wind tunnel studies, where attraction of *C. pronubana* male moths occurred with a wide range of Z9/ $\Delta 11$ ratios (Witzgall, 1989). Likewise, Z9/Z11-14:Ac ratios varied widely in *Adoxophyes orana* females (Guerin et al., 1986). This suggests that production of Z9-14:Ac is controlled independently from the $\Delta 11$ -14-components. In *Trichopusia ni* production of Z9-14:Ac and Z11-14:Ac differs in at least terminal steps (Bjostad et al., 1984).

The importance of specific E11/Z11 ratios for the reproductive isolation of tortricid species is now well established. The "list of sex pheromones of Lepidoptera and related attractants" (Arn et al., 1986) cites 52 chemically identified tortricid sex pheromones containing Z11-14:Ac, 37 of which contain, in addition, E11-14:Ac. So far, there are only five tortricid species sharing all three $\Delta 11$ -components (E11/Z11-14:Ac and Z11-14:OH) with *C. pronubana*. Emission of these compounds in constant proportions should ensure reproductive isolation of the day-active carnation tortrix; interindividual variation of $\Delta 11$ -14:Ac/Z9-14:Ac ratios accounts for a diversity of the chemical signal that might play a role in intraspecific communication.

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PUNGENT SPICES, GROUND RED PEPPER, AND SYNTHETIC CAPSAICIN AS ONION FLY OVIPOSITIONAL DETERRENTS¹

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Abstract—In laboratory choice experiments, the spices dill, paprika, black pepper, chili powder, ginger, and red pepper deterred *Delia antiqua* oviposition by 88–100%. Dose–response choice tests demonstrated that 1 mg of ground cayenne pepper (GCP) placed within 1 cm of artificial onion foliage reduced oviposition by 78%. A synthetic analog of capsaicin, the principal flavor ingredient of red peppers, deterred oviposition by 95% when present at 320 ppm in the top centimeter of sand (the ovipositional substrate). However, in no-choice conditions 10 mg GCP was not an effective deterrent. Sevana Bird Repellent and Agrigard Insect Repellent both use red pepper as a principal ingredient; at recommended field rates, neither of these materials was an effective ovipositional deterrent either in laboratory or field. Capsaicin-based materials do not appear to be candidates for onion maggot control via behavioral modification.

Key Words—Onion fly, *Delia antiqua*, Diptera, Anthomyiidae, oviposition, deterrent, capsaicin.

INTRODUCTION

In accepting hosts, insects integrate, via multiple sensory modalities, a diversity of external excitatory and inhibitory inputs with internal excitatory and inhibitory inputs, that establish internal “physiological status” (Dethier, 1982; Miller and Strickler, 1984). As for many insect herbivores, external excitatory inputs affecting onion fly oviposition have been investigated much more extensively

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(Harris et al., 1987; Harris and Miller, 1982, 1983, 1984), than external inhibitory inputs.

We investigated pungent spices as ovipositional deterrents of *D. antiqua*. After ascertaining that various spices deter *D. antiqua* oviposition in choice tests, ground cayenne pepper was chosen for detailed study involving both choice and no-choice conditions because: (1) a synthetic analog of the principal flavor ingredient was readily available, (2) commercial products, including an insect repellent mixture available for field use, contain capsicum oleoresin as a principal ingredient, and (3) at least in vertebrates, capsaicinoids cause stimulation of chemoreceptors and heat and pain receptors (Virus and Gebhart, 1979), making animal behavioral responses likely.

METHODS AND MATERIALS

D. antiqua *Culture*. A laboratory culture was started from pupae collected from onion culls left in harvested fields in Grant, Michigan, in September 1986. Adults were housed in 80 × 65 × 60-cm screened cages provisioned with water, food (Ticheler, 1971), and ovipositional resources and were held at 16:8 light-dark photoperiod, 21 ± 1°C, and 70 ± 5% relative humidity. Larvae were reared on bisected onions. Flies used in all experiments were three to five generations removed from the field.

Laboratory Ovipositional Bioassays. Ovipositional assays were conducted in a second walk-in environmental growth chamber having conditions identical to the rearing chamber except this room was free of onion volatiles. All choice experiments were conducted in 57-cm-diam. × 57-cm-tall cylindrical cages stocked with ca. 50 male and 50 female flies provisioned with diet (Ticheler, 1971) and water. These cages rotated once every 13 min, thus experimental error was minimized by distributing environmental gradients evenly over all treatments (Weston and Miller, 1985).

Ovipositional dishes consisted of 50 g white silica sand (Unimin Granusil, Grade 40, Oregon, Illinois) moistened with 3 ml distilled water, tamped into a 4-cm-diam. × 4-cm-tall plastic cups. Standing upright in the center of each dish was one of the Harris et al. (1987; Harris and Miller, 1982, 1983, 1984) surrogate onions consisting of an onion-green 4-mm-diam. × 12-cm-long glass tube coated with paraffin wax containing 0.05% *n*-dipropyl disulfide. The surrogate onion was swiveled in the sand to provide a 1-mm space for ovipositor probing. This surrogate onion foliage has been shown to be competitive with similarly sized onion foliage (Harris et al., 1987), and offers a highly controlled ovipositional resource for testing putative deterrents. Since critical host examining behaviors and oviposition mostly occur within 1 cm of the juncture of

foliage and soil (Harris et al., 1987), deterrents should be assayed most effectively by placing them in this region.

With the exception of the no-choice experiment, all tests were randomized complete-block designs, with treatments placed 5 cm from the perimeter of the cage in randomized order and blocked over time intervals, usually by day. Replicates with combined treatment counts of less than 50 eggs were pooled to minimize sampling error. Egg counts were not distributed normally; however, log-transformed $[\ln(x + 1)]$ counts fulfilled assumptions for analysis of variance. A two-way analysis of variance general linear models procedure (SAS Institute, Cary, North Carolina) was used, and means were separated with the Student-Newman-Keuls' test for multiple comparisons.

Botanicals. Pungent spices were presented as choices in one cage, along with a foliar surrogate control. Treatments consisted of 5–7 mg of each of the following spices scattered within 1.5 cm of the surrogate onion foliage: crushed red pepper, consisting of 3×5 -mm flakes with seeds; chili powder, containing chili pepper, onion, cumin, garlic, oregano, cayenne pepper, black pepper, caraway and silicon dioxide; dill weed, flakes 1 cm \times 1 mm; ground ginger; and coarsely ground black pepper (R. T. French Co., Rochester, New York 14692). This experiment was replicated three times, and flies were allowed to oviposit 5, 6, and 48 hrs., respectively, for each replicate.

Dose-Response Series. Dose-response choice tests were conducted simultaneously in three cages, each cage with various concentrations of one test material. Ground cayenne pepper (GCP) (McCormick & Co., Baltimore, Maryland) or Sevana Bird Repellent (SBR) powder (Sevana Co., Fresno, California) were applied in quantities of 0 (control), 1, 2, 5, 10, 22, and 46 mg, placed within 1 cm of the surrogate onion. Agrigard Insect Repellent (AGR) (Sevana Co., Fresno, California) diluted to 1, 3.2, 10, 32, 100, and 320 ppt in deionized distilled water was sprayed (ca. 0.175 ml) on the sand surface with a TLC atomizer.

Ovipositional deterrence of synthetic capsaicin was assayed in a dose-response choice test with 50 males and 50 females per cage. Synthetic capsaicin (97% *n*-vanillyl-*n*-nonamide, Pfaltz & Bauer, Waterbury, Connecticut) was dissolved in 95% ethanol and diluted to 6.32, 20.0, 63.2, 200, 632, 2000, and 6320 ppm. Twenty milliliters of each solution was added to 200 g white silica sand, mixed thoroughly, and allowed to air dry. Final concentrations were 0.316, 1.00, 3.16, 10.0, 31.6, 100, and 316 $\mu\text{g/g}$ sand. Each sand treatment was moistened with 10 ml deionized distilled water and added in a 1-cm layer on top of clean sand in oviposition cups.

No-Choice Test. GCP was assayed in a no-choice context that employed a 2×2 factorial, completely randomized design that quantified effects of exposures during both prereproductive and reproductive periods. Prereproductive

exposure was effected by placing 120 flies (not sexed, less than 24 hr posteclosion) for five days in a cage with food, water, and a foliar surrogate treated with 10 mg GCP. A second group received similar treatment but no GCP. Females from these two groups were transferred individually to 9-cm-diam. screen-sided cages and provided with two males, food, water, and an ovipositional dish. Half the females experiencing GCP prereproductively were provided standard foliar surrogate; half were provided foliar surrogate plus 10 mg GCP. Similar reproductive exposure treatments were provided to females that had not experienced GCP.

Egg counts were taken daily from day 6 to day 15 posteclosion to measure days until first oviposition as well as daily oviposition. Onion flies in this experiment tended to lay most eggs on alternating days, which hindered data analysis because of zero counts. This problem was eliminated by pooling counts every two days. Egg counts for flies that died during the experiment (18% of total flies) were not used in the analysis; however, their ovipositional record was included when comparing days until first oviposition. Days to first oviposition data could not be analyzed by parametric methods due to nonhomogeneity of variance. These data were therefore analyzed with the Kruskal-Wallis test (Steel and Torrie, 1980); treatment comparisons were then conducted with the Wilcoxon-Mann-Whitney test (Steel and Torrie, 1980).

We hypothesized that if habituation or adaptation to GCP had occurred in the no-choice experiment, then flies with the greatest exposure would show diminished GCP deterrence in a follow-up choice test, e.g., when compared to flies not exposed or exposed only prereproductively. Therefore, groups of three females were pooled from each treatment and placed in 9-cm-diam. screen cages with food, water, and two oviposition cups, one with 10 mg GCP, one without. The percent ovipositional deterrence was then compared for the four treatment groups.

Field Trials with Capsaicin-Based Products. Field trials of AGR and SBR ovipositional deterrence to *D. antiqua* were carried out in 1986 in a commercial onion field (Eaton Rapids, Michigan) with historically moderate onion fly population pressure. Plots consisted of 3 m of treated row, separated by 3 m of buffer row, in a linearly arranged randomized complete-block design replicated six times. Blocks were separated by ca. 35 m. SBR treatments were 0.05, 0.5, or 5 g/plot, applied to the soil at the base of onion plants with salt shakers. AGR treatments were 1.5 mg, 15 mg, 150 mg, 1.5 g or 15 g plus 15 mg Vaporguard (Miller Chemical Co., Hanover, Pennsylvania), in 70 ml distilled water per plot, using Chapin compressed air sprayers (Model 110, R.E. Chapin Manufacturing Works, Inc., Batavia, New York 14020), with Teejet 730077 fan nozzle at 20 psi (Spray Systems Co., North Ave, Wheaton, Illinois 60188). Negative controls for these treatments were 70 ml water and 15 mg Vaporguard in 70 ml water applied in 3-m plots in each block. There were nine applications

made of SBR and AGR, on a 7- to 10-day schedule starting May 27. Damage estimates in conventionally pesticide-treated rows (liquid chlorpyrifos, ca. 1.1 kg AI/hectare) were made in 3 m of row 1 m laterally from untreated plots. Stand counts were made May 27 and at two-week intervals. Analyses were conducted on percent damage [(100 × stand count on August 6)/stand count May 27]; these data did not require transformation.

RESULTS AND DISCUSSION

Botanicals. In choice experiments, pungent spices all significantly deterred onion fly oviposition (Figure 1). Mean percent ovipositional deterrence of these commercial spices was: paprika (88.6%), red pepper (95.9%), ginger (99.0%), dill (99.3%), chili powder (99.8%), and black pepper (100%).

Black pepper, red pepper, and ginger were pungent spices that showed great promise in choice tests. Unfortunately, the pungent flavor components of black pepper, predominantly piperine, are suspected carcinogens (Buchanan, 1978), while gingerol, the pungent principle ingredient of ginger (Wildholz, 1976), is relatively expensive. Red pepper was chosen for further studies based on its relatively well-known physiological effects on taste perception in mam-

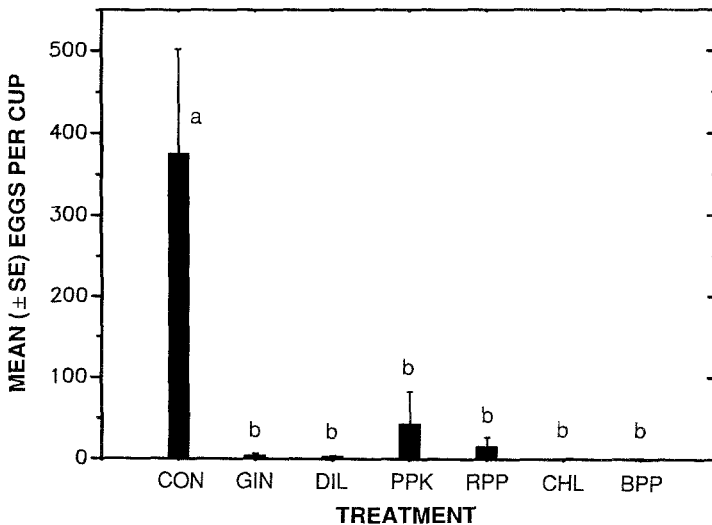


FIG. 1. Number of eggs laid with 5–7 mg of the following spices placed within 1.5 cm of surrogate onion in choice tests: CON, control; GIN, ginger; DIL, dill; PPK, paprika; RPP, red pepper; CHL, chili powder; BPP, black pepper. Means followed by the same letter are not significantly different (SNK test, 2 *df*).

mals and the availability of synthetic capsaicin. Capsaicinoids, the pungent principal flavor ingredients in red pepper, cause bursts of activity from contacted chemoreceptors and nociceptors (Virus and Gebhart, 1979). Capsaicinoids have some similarities to warburganal, a potent *Spodoptera exempta* antifeedant from the bark of an African tree (Nakanishi, 1980). Both capsaicin and warburganal have a pungent flavor and are used as spices (Todd et al., 1977; Nakanishi, 1980); in both cases these chemicals cause rapid firing from chemoreceptors that then become unresponsive to stimulation (Virus and Gebhart, 1979; Ma, 1977). It may be that such nonspecific activity at the level of gustatory chemoreceptors enables these spicy substances to interfere with normal host acceptance behavior.

Dose-Response Series. In dose-response choice tests, oviposition of onion flies was reduced 78-99% by the presence of 1-46 mg GCP (Figure 2) ($F = 26.1$; $df = 6, 24$; $P < 0.001$). Increasing quantities of GCP clearly caused greater reductions in the number of eggs laid next to treated surrogate foliage. Agrigard deterred oviposition ($F = 10.0$; $df = 6, 36$; $P < 0.001$) at the highest rates tested, with 98% deterrence at 100 ppt and 100% deterrence at 320 ppt (Figure 3); recommended field rates correspond approximately to 32 ppt, which was not significantly different from the control. Sevana Bird Repellent showed no deterrence, even at the highest rate tested ($F = 0.58$; $df = 6, 36$). Synthetic

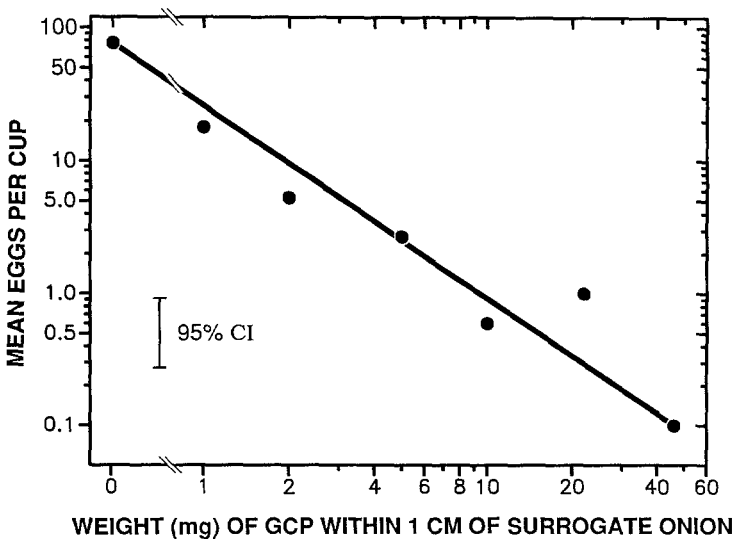


FIG. 2. Dose-response relationship for oviposition when ground cayenne pepper was placed in choice tests within 1 cm of surrogate onion foliage. Straight line fit by eye. Confidence interval based on 7 df .

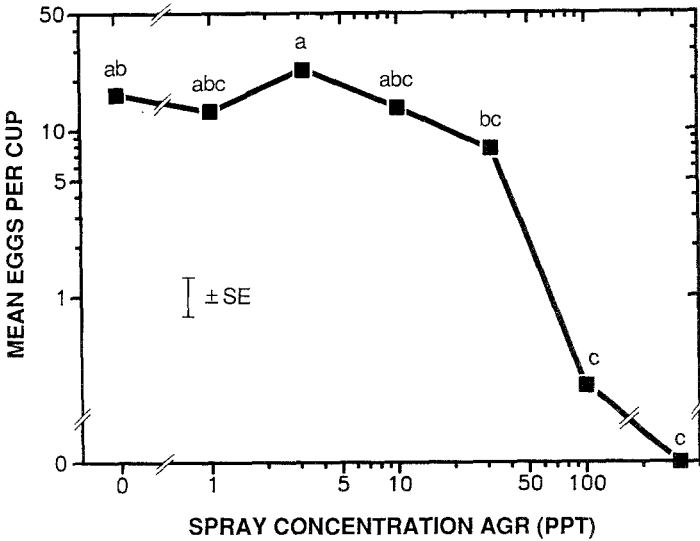


FIG. 3. Dose-response curve for oviposition when substrate was sprayed in choice tests with 0.175 ml of Agrigard Insect Repellent solutions. Standard error based on 6 *df*.

capsaicin incorporated into the top 1 cm of ovipositional substrate significantly deterred oviposition when present at concentrations greater than 600 ppm ($F = 35.4$; $df = 7, 77$) (Figure 4).

As summarized by Dethier et al. (1952), many chemicals are deterrent or repellent at high concentrations, including host-plant chemicals, which at lower concentrations are involved with host acceptance. These dose-response choice tests were conducted to be certain that low rates of a promising material did not stimulate oviposition. Any material showing stimulatory activity at low concentrations would cause logistical problems when applied in the field, because chemical decomposition would eventually decrease the concentration to levels that could stimulate insect damage. Dose-response experiments with capsaicin-based products elicited no increased oviposition through the range of rates tested, unlike dipropylsulfide, which shows deterrent activity at high concentrations (Matsumoto and Thorsteinson, 1968) and stimulatory activity with an optimum concentration in surface wax of about 0.05% (Harris et al., 1987). Direct visual observations of fly behavior suggested that, even at the highest rates tested, onion flies did not orient away from GCP; so, the end result appears to be mediated by deterrence upon contact rather than repellency.

No-Choice Test. In the no-choice experiment, there was no evidence that GCP deterred oviposition (Figure 5). Prereproductive exposure showed no significant effect on two-day egg counts ($F = 0.19$; $df = 1, 39$); exposure during

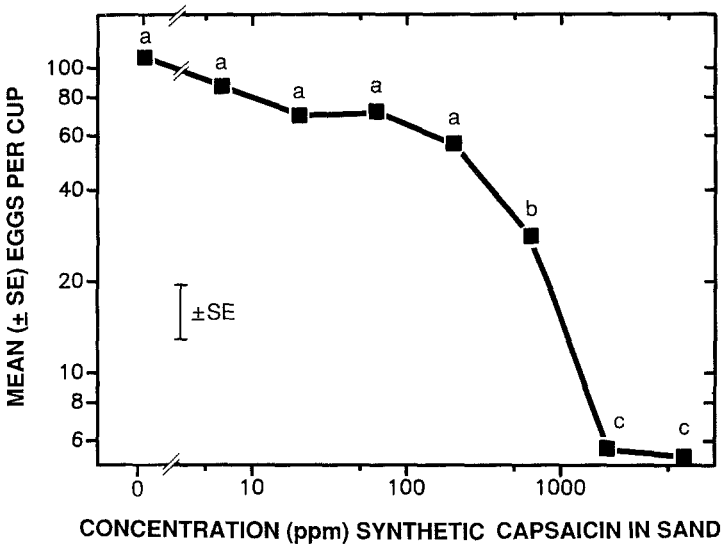


FIG. 4. Dose-response relationship for oviposition with synthetic capsaicin placed within the top centimeter of sand.

peak reproductive activity also was not significant ($F = 0.39$; $df = 1, 39$) (Figure 5). There were significant differences between treatments in days to first oviposition (Kruskal-Wallis test, $H/D = 11.25$, $P < 0.025$) (Steel and Torrie, 1980). Multiple comparison of treatments showed only one significant difference, flies not exposed to GCP laid eggs sooner (mean of 6.9 days) than flies of the reproductive-exposure-only treatment (mean of 7.8 days) (Wilcoxon-Mann-Whitney test, $T' = 89.5$, $n_1 = 10$, $n_2 = 14$, $P < 0.05$) (Steel and Torrie, 1980).

No-choice tests of deterrents are a rigorous test of deterrence, because insects under these conditions face increasing internal excitatory inputs that can override the presence of external inhibitory inputs. No-choice situations may simulate some field situations (where alternative acceptable ovipositional substrates may not be available), and also assist in determining whether choice test deterrence is caused predominantly by sensory vs. sublethal physiological effects (Landis and Gould, 1989). Our no-choice tests effectively ruled out the possibility that effects of GCP seen in choice tests were caused by sublethal toxicity, since all no-choice groups laid similar numbers of eggs. This is in contrast to preliminary screening with pyrethroids, in which "deterrent" concentrations were accompanied by convulsions or tremors in onion flies (unpublished data).

In the choice test bioassay of flies previously used in the no-choice experiment, all groups laid similar percentages of eggs on the control ($84 \pm 2.7\%$,

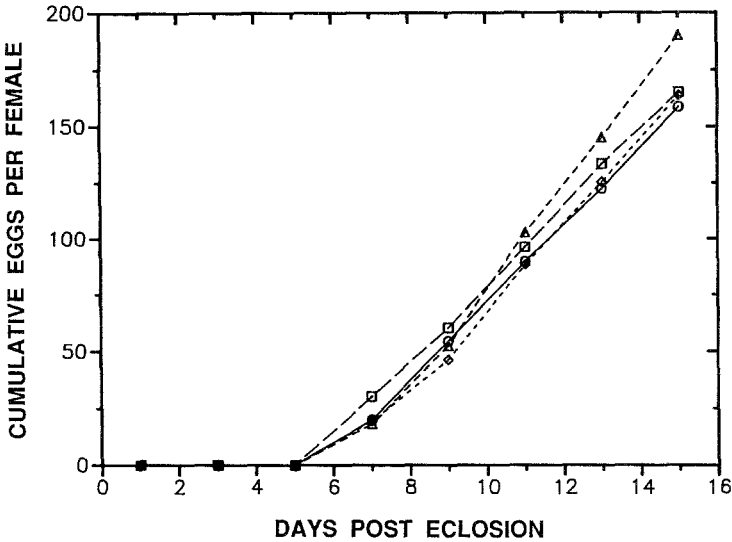


FIG. 5. Cumulative oviposition for 2×2 factorial experiment investigating prereproductive and reproductive exposure to GCP. ---◇---, no exposure; ---□---, exposure only prereproductively; ---△---, exposure only reproductively; —○—, continual exposure.

mean \pm SE) ($F = 0.49$, $df = 3, 6$), implying that these groups retained the same ability to sense and respond to the presence of GCP. Whether habituation or adaptation were responsible for the lack of differences in the no-choice experiment is still unclear; either dishabituation occurred rapidly or another mechanism was responsible for acceptance of GCP-treated ovipositional substrates. Perhaps flies simply tolerated deterrent because they were becoming deprived.

Field Trials. Field tests of AGR and SBR in 1986 for the most part agreed well with laboratory studies; however, the trends in field data were not statistically significant. There was a trend toward higher mean damage for all three rates of SBR in the field than in the control plots (Figure 6). These field results suggested that SBR may have stimulated oviposition, in contrast to the lack of response (stimulatory or deterrent) to SBR in the laboratory. Under the wet field conditions experienced in 1986, there exists the possibility that SBR, which consists of 10% ground red peppers and 4% ground garlic, could have decomposed to form microbial products stimulatory to onion fly oviposition (Coley-Smith and King, 1969; Dindonis and Miller, 1980).

The most highly concentrated sprays of AGR suppressed damage to a level intermediate to the untreated controls and the conventionally pesticide-treated areas. These sprays were irritating to those applying them, and at elevated rates

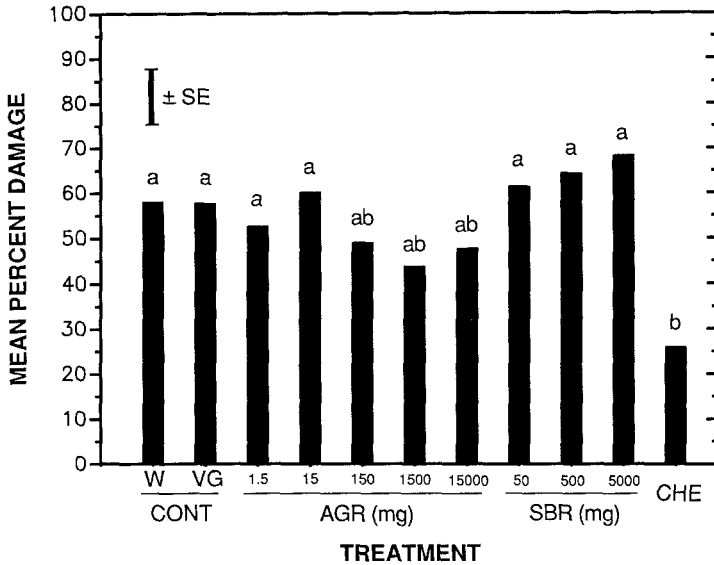


FIG. 6. Percent damage to seedling onions by onion fly. W, water control; VG, Vapor-guard control; AGR, Agrigard; SBR, Sevana Bird Repellent powder; CHE, conventional chemical control (0.05 g chlorpyrifos/m). Amounts given are for 3 m of row. Means followed by the same letter are not statistically different (SNK test, 5 *df*).

caused onions to have stunted, yellow foliage. These field results with AGR agree with the laboratory studies that indicated that ovipositional deterrence only occurred at concentrations exceeding the field recommended rates.

General Discussion. Ovipositional and feeding deterrents should be tested with hosts or host models that accurately reflect normal sensory input (Städler, 1983). Using natural stimuli allows normal sequences of behaviors to proceed. Allowing the full repertoire of behaviors involved with host acceptance to be expressed implies that each behavior has an opportunity to be influenced by the presence of deterrents. When using highly artificial substrates, internal excitatory inputs may override inputs associated with normally nonstimulatory substrates to provide abnormal behavioral responses. An example demonstrating how ovipositional substrates can influence results while studying ovipositional deterrents is the recent work of Tingle and Mitchell (1986). Their data show that in choice tests, elderberry extracts were more deterrent to *Heliothis virescens* when applied to tobacco (a preferred host) compared to a standard paper towel substrate.

External excitatory inputs for the onion fly, *Delia antiqua* (Meigen), involve synergism between visual and chemical stimuli (Harris and Miller, 1982). Yellow or green vertical cylinders ca. 4 mm diam. that emit *n*-dipropyl disulfide from surface waxes elicit preovipositional behaviors, including foliar

runs, ovipositor examining of foliage and soil, and ovipositor probing (Harris et al., 1987). These surrogate onions provided a highly standardized ovipositional resource that accurately simulated host stimuli. We suggest they are highly suitable and convenient for studying ovipositional deterrence.

Ovipositional deterrents have not previously been tested for the onion fly with host models. Wiens et al. (1978) were able to reduce oviposition by ca. 78% over controls in choice tests using hydrated bean (*Phaseolus vulgaris*) extracts. Alfaro et al. (1981), using cedar (*Thuja plicata*) leaf oil, found an 84% ovipositional deterrence. Both of these experiments used inverted beaker ovipositional bioassays (Vernon et al., 1977). Deterrence assays with pine oil (Javer et al., 1987) similarly used halved onion bulbs rather than onion foliage or foliar models. Irrespective of bioassay method, results to date suggest that a wide variety of compound classes can appreciably deter onion fly egg laying, and some, such as pine oil at dosages of 5 mg/onion, deter appreciably in the no-choice situation.

While pungent spices appear to be highly deterrent materials for onion fly oviposition in laboratory choice tests and could perhaps be of some use in the small home garden, we do not feel that they hold much promise for commercial field use. The poor results in 1986 field tests may have been due partially to extremely wet weather or to the lack of a highly acceptable alternative ovipositional resource (trap crop). A follow-up test in 1987 (unpublished data) suggested that poor deterrent activity probably was not due to the lack of availability of suitable ovipositional resources. Materials that are both less costly and show greater activity in both choice and no-choice tests are currently being investigated.

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DEFENSIVE SESQUITERPENOIDS FROM A DIPTEROCARP (*Dipterocarpus kerrii*)¹

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Abstract—Four sesquiterpenoids (**2**, **4**, **7**, and **9**) have been isolated and characterized from the termiticidal fraction of *Dipterocarpus kerrii* resin. The major constituent of this resin is α -gurjunene (**1**).

Key Words—Resins, *Dipterocarpus kerrii*, termite, sesquiterpenes, α -gurjunene, epicyclocolorenone, fungicidal, termiticidal, bioassay.

INTRODUCTION

Trees of the plant family Dipterocarpaceae dominate many lowland primary forests of Southeast Asia. Often attaining a height of 70 m or more, these hardwoods can comprise 80% of the emergent vegetation in some areas (Ashton, 1982). With species richness at a maximum on the Indonesian island Kalimantan, Dipterocarpaceae range from Africa to Papua New Guinea. Because of their abundance and durability, these timbers have become important economic commodities.

Southeast Asian dipterocarps produce copious amounts of resins (Ashton, 1982; Torquebiau, 1984). Exuded from natural or artificially induced trunk wounds, these viscous and sticky resins have long been items of commerce

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(Marsden, 1783). Dipterocarp resins contain a large and complex array of chemical constituents (Hegnauer, 1966; Bisset et al., 1971; Ashton, 1982), many of which are terpenes. The diversity of terpene and other fractions indicates that dipterocarps dedicate a large number of biosynthetic pathways to the production of resin chemicals. These biosynthetic pathways must be metabolically costly, but in the absence of data on biological roles of dipterocarp resins, the adaptive benefits (if any) of maintaining these pathways remain obscure.

Several lines of evidence suggested that dipterocarp resins might contain biologically active molecules. Considering evidence scattered throughout the literature of Asian forestry, we postulated that some of the chemicals made by dipterocarps might play a defensive role. Dipterocarp timbers are well known to resist biological attack from many sources. *Shorea robusta* was shown highly resistant to the termites *Microcerotermes beelsoni* and *Heterotermes indicola* (Sen-Sarma, 1963; Sen-Sarma and Chatterjee, 1968). Particle boards constructed from *Shorea* species are also protected against *Cryptotermes cynocephalus* (Moi, 1980). Fresh resins of *Anisoptera thurifera* appear to protect bee nests from termites (Messer, 1984).

Dipterocarp woods cause substantial mortality to insects feeding on them. Over a three-month test period, termites feeding on *Shorea* species suffered 99% and 86% mortality, while termites feeding on the nondipterocarp *Dyera costulata* showed only a 13% death rate (Moi, 1980). Tested as a possible substrate for insect culture, dipterocarp sawdust killed stable fly larvae in the first instar, while other woods did not (Sutherland, 1978).

Chemical factors in resins may also protect dipterocarps from microbial attack. Untreated dipterocarp timbers are reported to be highly resistant to fungal invasion (Bakshi et al., 1967), and volatile components of *Hopea papuana* were shown to inhibit fungal growth (Messer, 1985). Bacterial growth inhibitors of dipterocarp origin include essential oils of *Vateria indica* (Bhargava and Chauhan, 1968) and stemnopolol and alpha-copalliferol from Sri Lankan dipterocarps (Sootheeswaran et al., 1983).

The research described here tested the hypothesis that dipterocarp resins contain biologically active components that protect the trees against insects and fungi. Antifungal and termiticidal properties of whole resins and resin fractions were evaluated, and novel compounds possibly mediating the observed biocidal properties were isolated and characterized.

METHODS AND MATERIALS

Tree Resins

Fresh resins of *Dipterocarpus kerrii* King were collected by tapping trees cultivated at the Forest Research Institute, Kepong, Malaysia. Resins were stored in sealed glass ampoules for transport to the United States.

Termite Bioassays

Zootermopsis angusticollis (Hagen) termites were taken from a permanent laboratory culture. Termites selected for bioassay experiments were undifferentiated larvae ("workers") beyond the third instar, and weighed 10–15 mg. Ten termites were placed in a 5-cm plastic Petri dish containing a 4.5-cm-diameter filter paper treated with compounds of interest. Treatment of filter papers involved uniform application of a methylene chloride solution of the chromatographic fractions of interest, followed by evaporation of the solvent. Three to four duplicate dishes were used for each condition. Mortality was checked at 24-hr intervals, and dead termites were removed.

Pilot experiments established that termite mortality was dose dependent. All filter papers were thus treated with the amount of a compound normally found in 10 mg of crude *D. kerrii* resin.

Fungal Bioassays

Ten milligrams of crude *D. kerrii* resin dissolved in methylene chloride was applied to a 10 × 20-cm polyester TLC plate and eluted with 50% ether-hexane. After the solvents had evaporated, the plate was sprayed with a suspension of *Cladosporium cucumerinum* spores. The suspension was prepared by scraping spores from three confluent plates into ≈ 5 ml of sterile water, mixing this solution with 50 ml of 0.35% green bean juice agar at 37°C, and sonicating the solution for 3 min prior to spraying the TLC plates. Plates were examined for regions of antifungal activity after incubating for 48 hr at room temperature in TLC tanks lined with moist paper towels. Zones of fungal inhibition were apparent as white bands against the background of grey-green fungal growth.

Isolation

Crude *D. kerrii* resin was dissolved in methylene chloride and applied to 1000 μm preparative TLC plates (silica gel GF, Analtech, Inc., Newark, Delaware), which were eluted with 100% pentane or 50% ether-hexane. Component bands were visualized by means of a UV light and/or anisaldehyde spray reagent (90% of a 5% solution of anisaldehyde in 95% ethanol–5% acetic acid–5% conc. H₂SO₄). Bands of interest were removed from the plates, and the silica gel was repeatedly washed with methylene chloride, which was removed by evaporation in vacuo.

Further purification was effected by high-performance liquid chromatography (HPLC) using a Waters Associates M6000A solvent delivery system and a preparative scale column (20 mm ID × 250 mm) packed with 5-μm silica. The HPLC column was eluted with 5% ether-hexane at a flow rate of 1.5 ml/min. Ultraviolet detection was performed at 254 nm with a Perkin-Elmer model

LC-65T variable wavelength UV detector. Eluent from HPLC peaks of interest was collected and concentrated at ambient pressure using Kaderna-Danish (Ace Glass, Inc.) microscale concentrators.

Final purification of resin components was effected by semipreparative gas-liquid chromatography (GLC) with a Varian model 2100 gas chromatograph equipped with a flame ionization detector and a glass column (3 mm \times 1 m) packed with 3% OV-1 on 100/120 Supelcoport (Supelco, Inc.). Nitrogen was used as the carrier gas at a flow rate of 30 ml/min, and the column temperature was held at 150°C for 6.5 min, and then programmed to 200°C at 20°/min. The effluent from the column was split in a 1:9 ratio between the detector and 25-cm glass capillary collection tubes (Brownlee and Silverstein, 1968) that were cooled in a Dry Ice-acetone bath. Analytical GLC was performed with the aforementioned Varian system or a Shimadzu Mini-2 equipped with a fused silica capillary column (007-methylsilicone, 0.25 mm \times 15 m, Quadrex Corp., oven temperature 200°C isothermal, helium as carrier gas at a linear flow of 2 kg/cm), a capillary split injection system with a 100:1 split, and a flame ionization detector.

Identification

The components of interest were identified using a combination of gas chromatography-mass spectrometry [GC-MS, using both electron impact (EI) and chemical ionization (CI)], gas chromatography-Fourier transform infrared spectroscopy (GC-FTIR), ultraviolet (UV) spectroscopy, and Fourier transform-nuclear magnetic resonance (FT-NMR) spectroscopy. Both proton [^1H]- and carbon [^{13}C]FT-NMR were utilized.

Mass spectra were obtained on a Finnigan model 3300 mass spectrometer equipped with a Systems Industries model 150 data reduction system, or a Finnigan model 4500 GCMS system. EI mass spectra were obtained at 70 eV; CI mass spectra utilized methane as the reagent gas. High-resolution mass spectra (HRMS) were measured on an Associated Electronics Industries MS-902 spectrometer interfaced to a VG Micromass 2040 data reduction system.

Microscale trimethylsilylation of purified resin components was performed in methylene chloride solution with excess Sylon-BTZ (Supelco, Inc.) at room temperature (10 min). Portions of these solutions were analyzed by GC-MS (EI).

Infrared spectra were obtained in CHCl_3 solution using a Perkin-Elmer model 299B infrared spectrophotometer; GC-FTIR spectra were obtained on an IBM model 98 GC-FTIR system coupled to a Hewlett-Packard model 5790A capillary gas chromatograph (007-methylsilicone column, 0.25 mm \times 25 m, Quadrex Corp.).

[^1H]NMR spectra were obtained using either a Bruker model WM-300

(300 MHz) or a Varian model XL 400 (400 MHz) NMR spectrometer. [^{13}C]FT-NMR spectra were obtained using either the aforementioned Varian system (at 100 MHz), or a Jeol model FX-90Q (at 22.5 MHz) NMR spectrometer. NMR spectra were measured in CDCl_3 or deuteriobenzene, which were stored over anhydrous K_2CO_3 and passed over a column of neutral alumina immediately prior to use.

Ultraviolet spectra were measured in cyclohexane or 95% ethanol using a Hewlett-Packard model 8450A UV-visible spectrophotometer. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter.

RESULTS AND DISCUSSION

Fungal Bioassays

Several components of *D. kerrii* resin inhibited fungal growth, as shown in Figure 1. Strong inhibition was shown by compounds with $R_f = 0.04$ and $R_f = 0.11$, as well as by a broad band at $R_f = 0.45$. Compounds with $R_f = 0.22$ and $R_f = 0.35$ produced less intense fungal growth inhibition.

Termite Bioassays

Guided by the termite bioassay, initial fractionation of crude resin was performed by TLC on 1000 μm preparative plates which were eluted with 50% ether-hexane. Resolved components were bioassayed at levels that mimicked a 10-mg dose of crude resin. After three sequential fractionations, termiticidal activity was found in a single TLC band that was UV active and that stained intensely with anisaldehyde. This TLC band, comprising ca. 12% of the crude resin, had the same R_f (0.45) as that which displayed strongest inhibition in the fungal bioassays. Toxicity results are shown in Figure 2A.

Further fractionation of the TLC active zone using preparative HPLC yielded one minor peak (1) and two major peaks (2 and 3, Figure 3). The major peaks were isolated and each was found to represent $\approx 4\%$ of the crude resin. As shown in Figure 2B, peaks 2 and 3 displayed essentially equivalent toxicity when bioassayed at levels corresponding to the 10-mg crude resin dose level.

Final fractionation of HPLC peaks 2 and 3 was accomplished using semi-preparative GLC. HPLC peak 2 was found to contain two major components (A and B), as was HPLC peak 3 (C and D, see Figure 4). The major GLC peaks were isolated in the following quantities: A: 2 mg; B: 0.2 mg; C: 2 mg; D: 0.8 mg. All isolated GLC peaks were found to be $\geq 95\%$ pure upon reinjection by analytical GLC. These materials were extremely labile and exhibited significant decomposition, even when stored at -10°C . Decomposition was

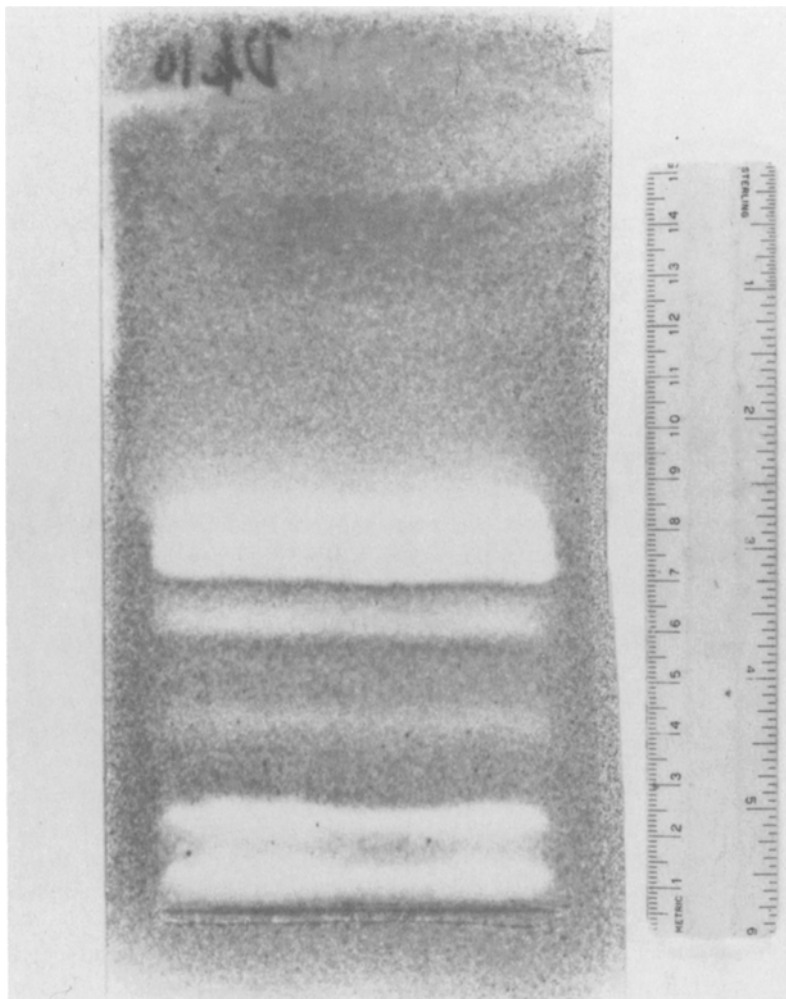


FIG. 1. Fungicidal bioautograph of crude *Dipterocarpus kerrii* resin against *Cladosporium cucumerinum*. Details in text.

exceptionally rapid in NMR solvents containing traces of acid (e.g., CDCl_3). As a result, these solvents were carefully pretreated with neutral alumina immediately prior to use. Due to these stability restrictions, and the quantities of materials needed for structure elucidation, bioassays of the individual components A, B, C, and D have not yet been completed.

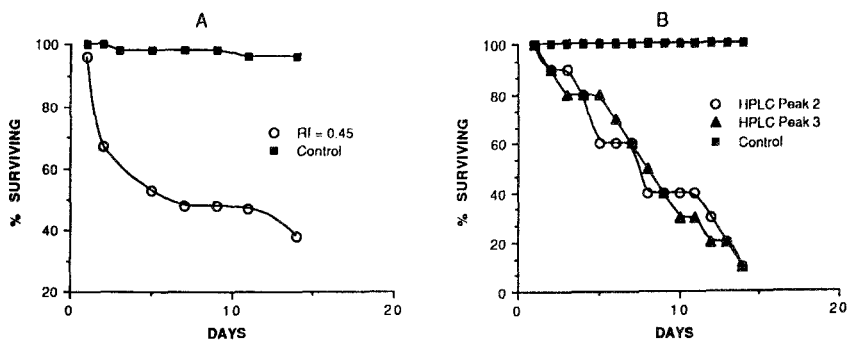


FIG. 2. Toxicity of *D. kerrii* resin components towards *Z. angusticollis*. (A) Preparative TLC band ($R_f = 0.45$); (B) preparative HPLC peaks 2 and 3. Details in Methods and Materials.

Isolation of Major Resin Component

In the preliminary stages of this study, examination of crude *D. kerrii* resin by GLC had shown the presence of a single major volatile component. Fractionation of resin by preparative TLC showed that the major volatile component corresponded to the most mobile TLC band, which was UV active and which

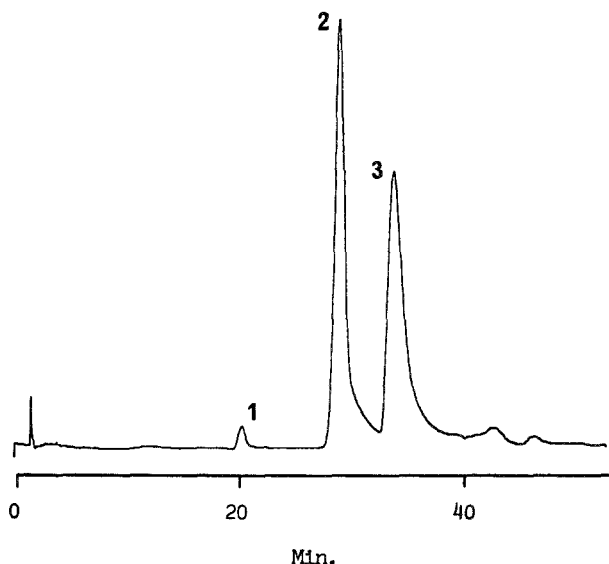


FIG. 3. High-performance liquid chromatogram of preparative TLC band ($R_f = 0.45$).

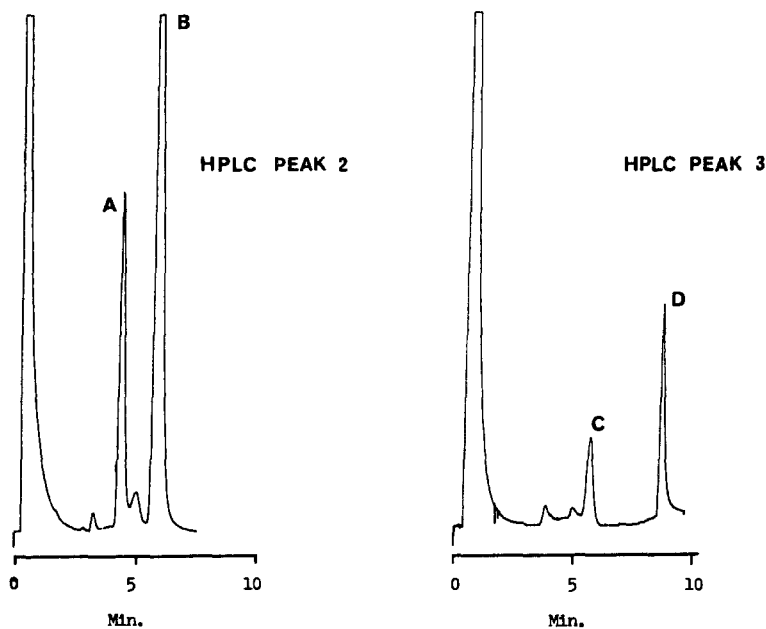


FIG. 4. Capillary GLC analysis of HPLC peaks 2 and 3.

stained intensely with anisaldehyde. Isolation of this component was accomplished by preparative TLC ($R_f = 0.95$, 1000 μm silica plate) using 100% pentane as eluent. This component, a colorless oil, comprised $\approx 24\%$ of the crude resin and was shown to be $\geq 95\%$ pure by analytical GLC ($R_t = 3.6$ min, 3% OV-1, 150°C). This material exhibited the same degree of lability as did the individual components A–D. Naturally, since this component did not elute with the TLC active zone, it did not display any toxicity in the bioassay.

Structural Analysis

Major Volatile Component. Analysis of the major volatile component by CI-MS established that the molecular weight of this material was 204. From HRMS analysis of the ion at $m/z = 204$, its molecular formula was found to be $\text{C}_{15}\text{H}_{24}$, indicating that this component was a sesquiterpene with four sites of unsaturation. The ^{13}C NMR spectrum of this material displayed a single pair of olefinic carbons (δ 137.26, 136.04) implying the presence of one double bond and three rings. Further, the ^1H NMR of this material exhibited three prominent methyl group signals [δ 0.84 (s), 0.91 (d), 1.66 (br.d)], and no olefin proton signals. Comparison of these data with information recorded in compilations of sesquiterpene data (Devon and Scott, 1972; Ourisson, 1966) allowed

the major volatile component to be identified as α -gurjunene, **1** (Figure 5). This compound, a member of the aromadendrane family of sesquiterpenes, was originally isolated from *Dipterocarpus dyeri* (Palmade et al., 1963) and has since also been found in resin from *Shorea flava* (Bisset et al., 1971). Our measured values for other physical properties of **1** (IR, UV, $[\alpha]_D$) were also in good agreement with the literature values (Palmade et al., 1963).

Peak A. Low-resolution EI-MS analysis of component A, a colorless oil, showed a very weak peak at $m/z = 220$ with diagnostic peaks at $m/z = 205$ [M-15 (CH₃)] and $m/z = 202$ [M-18(H₂O)], suggesting that A was a labile alcohol. The CI-MS spectrum of A also displayed a weak molecular ion signal at $m/z = 220$. HRMS analysis of this ion suggested the molecular formula of C₁₅H₂₄O. Finally, treatment of A with Sylon-BTZ followed by low-resolution GC-MS (EI) analysis showed quantitative conversion to a more volatile product with a molecular ion at $m/z = 292$. A gain of 72 mass units implied that A had been trimethylsilylated and strengthened the conclusion that A was an alcohol. This conclusion was also supported by observation of a weak OH band at 3637 cm⁻¹ in the GC-FTIR. The [¹³C]NMR spectrum of this material showed a single pair of olefinic carbons (δ 153.46, 106.25). Taken together with the HRMS data, this suggested that A was a tricyclic sesquiterpene alcohol with one double bond.

Comparison of this information, and extensive [¹H]NMR data, with liter-

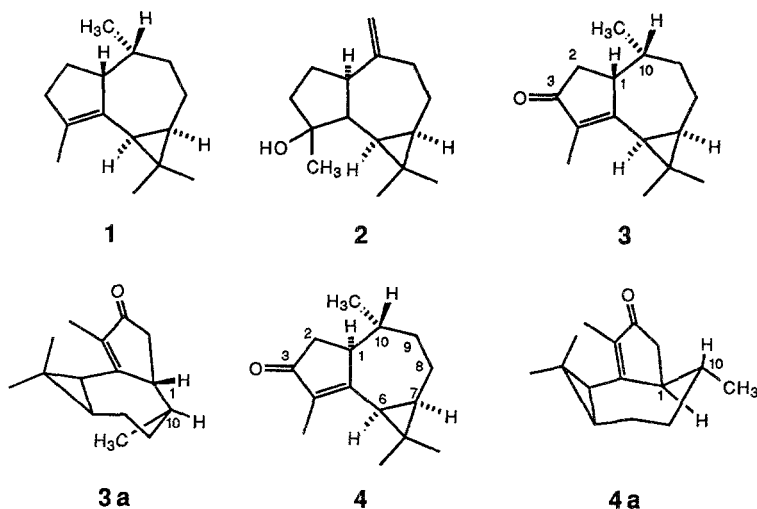


FIG. 5. Structures of previously identified sesquiterpenes isolated from *D. kerrii* resin, with the exception of cyclocolorenone, **3**, which has been isolated from *P. colorata*. Details in text.

ature data allowed identification of A as spathulenol, **2** (Figure 5). Like α -gurjunene, this material is an aromadendranoid sesquiterpene and was first isolated from *Eucalyptus spathulata* (Bowyer and Jefferies, 1963). In subsequent studies, spathulenol has been isolated from several other plant sources (cf. cotton plant, Elzen et al., 1984; *Citrus junos*, Shinoda et al., 1970). An especially extensive NMR/structural study of **2** was recently published (Inagaki and Abe, 1985). Our measured NMR data (conventional [^1H]- and [^{13}C]NMR spectra as well as two-dimensional [^1H]- and [^{13}C]NMR spectra) completely match these data.

Peak D. Analysis of D, also a colorless oil, by low-resolution CI-MS showed a molecular ion at $m/z = 218$. HRMS analysis of the $m/z = 218$ ion gave a molecular formula of $\text{C}_{15}\text{H}_{22}\text{O}$, implying that D was a sesquiterpene with five sites of unsaturation. GC-FTIR analysis showed an intense band at 1690 cm^{-1} and a moderately intense band at 1627 cm^{-1} , suggesting the presence of a conjugated enone; no OH band was observed. This was supported by the [^{13}C]NMR spectrum, which showed distinctive carbon signals at δ 200.96 (carbonyl), 141.60, and 99.47 (double bond).

Comparison of this information with tabulated sesquiterpene data (Devon and Scott, 1972; Ourisson, 1966) suggested that D was the known enone **3**, cyclocolorenone (Figure 5), first isolated from *Pseudowintera colorata* (Corbett and Speden, 1958). However, the UV data ($\lambda_{\text{max}} = 264\text{ nm}$; $e = 13,000$; EtOH) and optical rotation ($[\alpha]_{\text{D}} = -400^\circ$) reported for cyclocolorenone did not match our measured values: UV: $\lambda_{\text{max}} = 252\text{ nm}$ ($e = 4400$, EtOH); $[\alpha]_{\text{D}} = -200^\circ$ ($c \approx 0.8\text{ mg}$ in 5.0 ml , EtOH). These results lead us to conclude that D was actually **4** (Figure 5), the known C-1 epimer of cyclocolorenone. This compound, referred as epicyclocolorenone, has been obtained from **3** upon treatment with KOH (Corbett and Young, 1963) or upon chromatography on basic alumina (Büchi and Lowenthal, 1962). To the best of our knowledge, it would appear that our isolation of epicyclocolorenone from *D. kerrii* represents the first report of this material as a naturally occurring substance.

Our data for D correlated well with data reported for **4** (Büchi and Lowenthal, 1962; Corbett and Young, 1963; Büchi et al., 1966); UV: $\lambda_{\text{max}} = 253$ ($e = 9300$, EtOH); $[\alpha]_{\text{D}} = -167^\circ$ (EtOH). Especially diagnostic was the signal for the C-10 methyl group [δ 0.99 (d)] in the [^1H]NMR spectrum of D. As originally discussed by Büchi in his synthesis of epicyclocolorenone (Büchi and Lowenthal, 1962; Büchi et al., 1966), the C-10 methyl doublet in **4** is observed at δ 1.02, while in naturally derived cyclocolorenone this signal appears at δ 0.78. These chemical shift differences can be accounted for by consideration of the most stable conformations of cyclocolorenone and epicyclocolorenone (Büchi and Lowenthal, 1962; Büchi et al., 1966), as depicted in structures **3a** and **4a**, respectively (Figure 5). In **3a**, the seven-membered ring is in a pseudoboat form and the C-10 methyl group is in the shielding region of the extended

chromophore. In **4a**, however, the more stable conformer is the pseudochair form, which disrupts conjugation of the cyclopropyl group with the enone and places the C-10 methyl group in a pseudoaxial position. This accounts for the both the higher field position of the C-10 methyl in **4a** and for the large differences in the UV spectra of **3a** and **4a**.

Peak B. Low-resolution MS analysis of B, an extremely labile colorless oil, showed a very weak signal at $m/z = 220$ (both EI and CI), as well as moderately intense peaks at $m/z = 205$ [M-15(CH₃)] and 202 [M-18(H₂O)]. These data, like those for A, suggested that B contained a labile alcohol group. Also in common with A, GC-MS analysis of B after treatment with Sylon-BTZ showed quantitative transformation to a more volatile product with highest molecular weight ion at $m/z = 277$ [M(292)-15(CH₃)]. As before, this result suggested that B was an alcohol that had been trimethylsilylated. GC-FTIR analysis showed the presence of a very weak OH band at 3637 cm^{-1} and a moderately intense band at 1635 cm^{-1} (double bond). In analogy with data for A, the 3637 cm^{-1} IR signal implied that B was a tertiary alcohol. HRMS analysis of the B molecular ion gave a molecular formula of C₁₅H₂₄O. Taken together, these data suggested that B was a sesquiterpene alcohol with four sites of unsaturation.

The nature and number of the multiple bonds in B were deduced using several lines of information. First, because so little of B was isolated (0.2 mg), only a very poor [¹³C]NMR spectrum of this material could be obtained (20,000 transients). However, this spectrum showed four olefinic carbon signals: δ 149.1, 140.95, 131.32, 116.53 (d₆-benzene), implying the presence of two double bonds. Secondly, the [¹H]NMR spectrum (Figure 6) of B displayed two, one-proton olefin signals (δ 5.67, 5.40), which were not coupled, implying that both double bonds were trisubstituted. Finally, the UV spectrum of B showed $\lambda_{\text{max}} = 243\text{ nm}$ ($\epsilon \approx 2400$; cyclohexane), implying the presence of a conjugated diene. Hence, it was concluded that B was a bicyclic sesquiterpene alcohol containing a conjugated diene chromophore.

Compound B displayed four methyl group signals in its [¹H]NMR spectrum characterized as follows: δ 1.71 (m, olefinic methyl group); 1.225, 1.220 [singlets, implying two nearly equivalent methyl groups α to the tertiary alcohol moiety: C(CH₃)₂OH]; 0.70 (d, implying a CHCH₃ group). Accounting for the five carbons in these groups left 10 carbons to be distributed in the bicyclic system. Of the various possibilities, similarities between the [¹H]NMR spectra of B and compound **4** led us to begin with partial structure **5** (Figure 7). Thus, in compound **4**, the protons at position 2 appeared as a geminally coupled set with relatively low field positions [δ 2.58 (dd, $J = 6.4, 16.8\text{ Hz}$); 2.08 (br. d, $J = 16.8\text{ Hz}$)] due to the presence of an sp² center (carbonyl) at C-3. In B, a similar pair of signals was observed [δ 2.45, H_d (dd, $J = 7.8, 18\text{ Hz}$); 2.12, H_f (br. d, $J = 18\text{ Hz}$)] implying a similar arrangement of groups, in this case

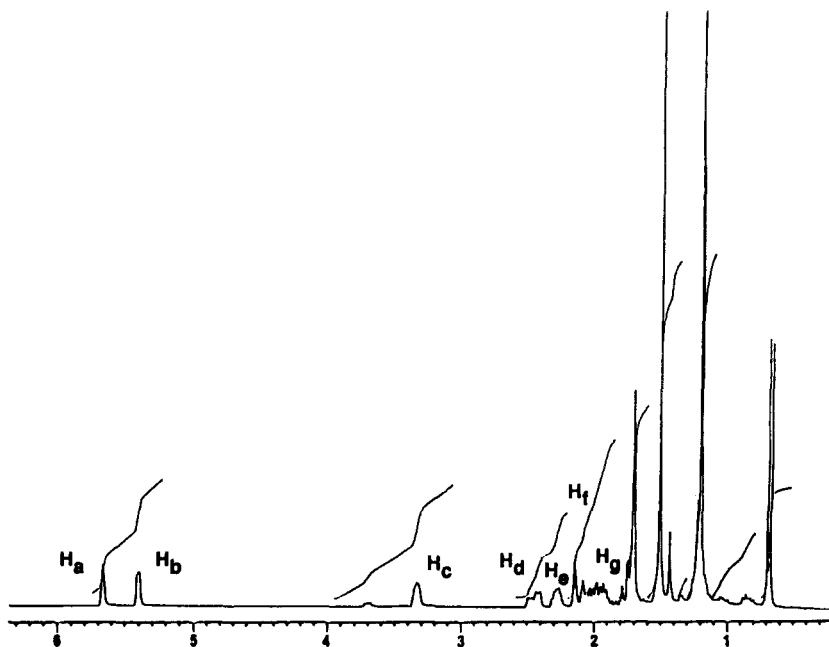


FIG. 6. 300-MHz PMR spectrum of compound B, identified as 7 (Figure 7).

with an olefinic carbon providing the sp^2 center at position 3. Very weak spin-spin coupling was observed between H_d and the olefin proton at δ 5.65 (H_a) and between H_a and the olefinic methyl group, indicating the double bond substitution pattern shown in 5. In addition, H_d was coupled ($J = 7.8$ Hz) to a methine signal at δ 3.34 (m, H_c) implying that the C-2 methylene was flanked by a bridgehead proton, as shown in 5.

Placing the remaining groups on the now required seven-membered ring in 5 was straightforward. Thus, methine proton H_c was coupled to a one-proton

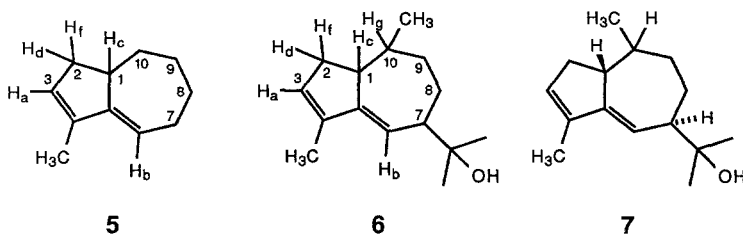


FIG. 7. Partial structures (5 and 6) and final structure for compound B, identified as 7.

signal at δ 1.93 (H_g) which was, in turn, coupled to the methyl doublet at δ 0.70. This implied that H_c was flanked by the $CHCH_3$ group. Finally, the unassigned $C(CH_3)_2OH$ group was placed in the remaining allylic position (C-7) giving structure **6** (Figure 7). This connection was consistent for several reasons: (1) it allowed assignment of H_e (δ 2.28, e.g., allylic and α to OH); (2) it corresponded to a conventional substitution pattern for a sesquiterpene; and (3) it correlated well with the observed lability of B (e.g., acid-catalyzed dehydration would produce a highly conjugated product). Hence, we assign structure **6** to B.

A computer search of the Chemical Abstracts On-Line data base for structure **6** produced a single entry, which has the stereochemistry shown in formula **7**. Ourisson reported the synthesis of **7** in the course of determining the structure of α -gurjunene, **1** (Streith and Ourisson, 1963). Oxidation of **1** with SeO_2 /acetic anhydride, followed by $LiAlH_4$ reduction, produced an "extremely labile compound," which was assigned structure **7**. Our measured values for B matched Ourisson's physical data for **7** well: UV: $\lambda_{max} = 243$ nm (cyclohexane); IR: 1630 cm^{-1} ($CHCl_3$); $[^1H]NMR$: δ 0.70 (d, 3H), 1.15 (s, 6H), 1.7 (m, 3H), 5.38 (1H), 5.6 (1H) ($CDCl_3$, 60 MHz). Our observation of **7** in *D. kerrii* resin represents the first isolation of this sesquiterpene from nature.

Peak C. Much of the chemical behavior of C in the early stages of analysis matched that of A and B. Thus, this material, also a colorless oil, displayed a very weak molecular ion at $m/z = 220$ and moderately intense peaks at $m/z = 205$ [M-15(CH_3)] and 202 [M-18(H_2O)]. GC-FTIR analysis showed a very weak OH band at 3741 cm^{-1} and a moderately intense band at 1643 cm^{-1} , implying the presence of a tertiary alcohol group and C-C double bonds, respectively. Low-resolution GC-MS analysis, after treatment with Sylon-BTZ, showed quantitative transformation to a more volatile product with a molecular ion at $m/z = 292$. Finally, HRMS analysis of the molecular ion for C once more indicated a molecular formula of $C_{15}H_{24}O$. In addition, C exhibited four olefinic carbon resonances in its $[^{13}C]NMR$ spectrum (δ 152.09, 145.83, 123.86, 110.96), and showed UV absorption ($\lambda_{max} = 219$ nm, $e = 1400$), implying the presence of a conjugated diene. Altogether, this information indicated that C was a bicyclic sesquiterpene containing a tertiary hydroxyl group and two conjugated double bonds.

Although rather complex, the $[^1H]NMR$ spectrum of C (Figure 8) displayed the following salient features: (1) three one-proton olefin signals [δ 5.71, H_a , dd ($J = 3.85, 6.7$ Hz, no coupling with other olefin signals); 4.77, H_b , br. s; and 4.68, H_c , br; coupling between H_b and H_c ($J \approx 2$ Hz), as well as the chemical shift and general appearance of these signals, implied that they represented a terminal methylene group]; (2) two downfield methine signals (δ 3.03, H_d , m; 2.85, H_e , m); and (3) three methyl group signals [δ 1.70, br. s (implying an olefinic CH_3 group); 1.27, s (implying a CH_3 α to the tertiary

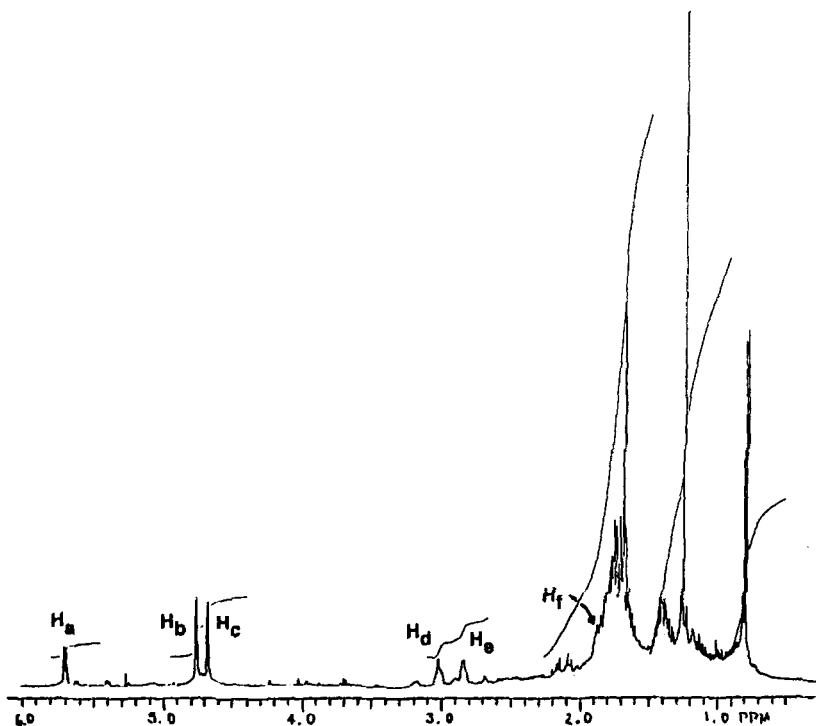


FIG. 8. 400-MHz PMR spectrum of compound C, identified as **9** (Figure 9).

hydroxyl group, as in **A**); 0.81, d (implying a CHCH_3 group)]. The remaining proton absorbances were located in two broad, complicated bands centered roughly at δ 1.75 ($\approx 5\text{H}$) and 1.4 ($\approx 4\text{H}$). Finally, by $[^{13}\text{C}]\text{NMR}$ analysis using an INEPT pulse sequence (Morris and Freeman, 1979; Doddrell and Pegg, 1980), **C** was found to contain three methyl (δ 27.27, 21.51, 14.76), five methylene (δ 110.96, 41.33, 32.86, 26.87, 25.33), four methine (δ 123.86, 46.65, 45.43, 32.86), and three quaternary (δ 152.09, 145.83, 80.74) carbon atoms.

Partial structure **8** (Figure 9) can be proposed for **C** on the basis of exhaustive decoupling studies (one- and two-dimensional), which yielded the following spin-spin coupling relationships. First, weak coupling between the olefin methyl group and the terminal methylene protons (H_b/H_c), together with a lack of coupling between H_a and H_b/H_c and the olefin methyl, suggested that the diene was composed of an isopropylidene group conjugated with a trisubstituted, endocyclic double bond. Secondly, **C** contained three nonolefinic methine carbons. One of these had to be present in the $-\text{CHCH}_3$ group. Indeed, irradiation at δ 1.89 (H_f , on the shoulder of the complicated band centered at δ

1.75) collapsed the methyl doublet at δ 0.70, establishing the chemical shift of this methine proton. Since C had been shown to be bicyclic, and since all of the quaternary carbon atoms had already been accounted for (olefins and tertiary alcohol), it was deduced that the remaining methines had to occupy bridgehead positions. The one-proton multiplets were obvious candidates for these methines. Thirdly, olefin proton H_a coupled to both of these methine signals (H_d , δ 3.03, $J = 3.8$ Hz; H_e , δ 2.85, $J = 6.7$), which were, in turn, coupled to each other. This implied that H_a , H_d , and H_e were on contiguous centers; the relative magnitudes of the H_a/H_d and H_a/H_e coupling constants indicated that H_e was the nearer to H_a . Finally, H_f coupled to H_d but not to H_e , while H_d was coupled to signals in the δ 1.85 region but not so for H_e . This implied that H_e was bordered by the remaining quaternary center, the tertiary alcohol, and that H_d was bordered by one of the unassigned methylene groups.

At this stage, only placement of the remaining four methylene groups was needed to complete a structure assignment for C. Of the various possibilities, a symmetrical distribution of the methylenes in the two rings of the bicyclic system was judged to best fit the $[^1H]NMR$, producing structure **9** (Figure 9). The nonsymmetrical distributions were ruled out as each would have produced highly distinctive features in the $[^1H]NMR$ that were not observed. In structure **9**, extensive coupling interaction between the methylene groups in the two sets would be expected to produce complex and broad $[^1H]NMR$ absorptions, just as is observed for C. To the best of our knowledge, this is the first report of structure **9** in the literature, constituting a new addition to the guiane family of sesquiterpenes. Studies to establish the stereochemical configuration of **9** are in progress.

What we can conclude from these studies is that the resin of *Dipterocarpus kerrii* contains small quantities of four labile sesquiterpenoids, closely related to α -gurjunene, which are responsible for the resin's termiticidal and antifungal activity. We hope to prepare each of these components in quantities sufficiently large to permit more detailed evaluation of their biological properties.

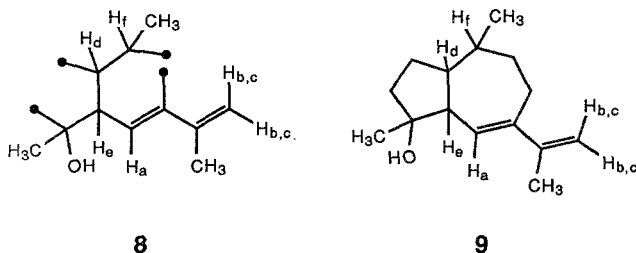


FIG. 9. Partial structure (**8**) and final structure for compound C, identified as **9**.

Experimental

Peak B. [^1H]NMR: δ 0.70 (d, 3H, $J = 7.3$), 1.22 (s, 3H), 1.225 (s, 3H), 1.24 (m, 2H), 1.71 (br. s), 1.76 (br. d, $J = 11.2$), 1.93 [m, 1H, $J = 7$ with C-10 CH_3 (δ 0.70)], 2.02 (m, 1H), 2.12 (br. d, 1H, $J = 18$), 2.29 (m, 1H), 2.45 (br. dd, 1H, $J = 7.8, 18$), 3.34 (m, 1H), 5.4 (br. d, 1H, $J = 3.9$), 5.67 (br. s, 1H). GC-FTIR: 3637 (v. weak), 1643 cm^{-1} . [^{13}C]NMR (d_6 -benzene, incomplete): 149.10, 140.95, 131.32, 116.53, 72.74, 52.36, 40.26, 34.70, 33.49, 32.42, 23.28, 12.36. UV (cyclohexane): $\lambda_{\text{max}} = 243 \text{ nm}$ ($e = 2400$). $[\alpha]_{\text{D}} = +50^\circ$ ($c \approx 0.2 \text{ mg}$ in 1.0 ml CCl_4). EI-MS m/z (rel. intensity): 220 (M+, 0.6), 205 (3), 202 (4), 187 (3), 162 (100), 161 (37), 147 (53), 133 (42), 120 (25), 119 (45), 107 (22), 105 (71), 94 (62), 91 (54), 81 (40), 79 (20), 59 (80). EI-MS (peak B + Sylon-BTZ) m/z : 277 [M-15(CH_3)]. HRMS: calcd for $\text{C}_{15}\text{H}_{24}\text{O}$: 220.1827; found: 220.1848.

Peak C. [^1H]NMR: δ 0.81 (d, 3H, $J = 7.2$), 1.27 (s, 3H), 1.28–1.5 (br. m, 4H), 1.70 (br. s, 3H), 1.40–1.83 (br. m, 5H), 1.89 [m, 1H, $J = 7$ with C-10 CH_3 (δ 0.81)], 2.85 (m, 1H), 3.03 (m, 1H), 4.68 (br. s, 1H), 4.77 (br. s, 1H), 5.71 (dd, 1H, $J = 3.85, 6.7$). GC-FTIR: 3741 (v. weak), 1643 cm^{-1} . [^{13}C]NMR: δ 152.09 (s), 145.83 (s), 123.86 (d), 110.96 (t), 80.74 (s), 46.65 (d), 45.43 (d), 41.33 (t), 32.86 (overlapping d and t), 27.27 (q), 26.87 (t), 25.33 (t), 21.51 (q), 14.76 (q). UV (cyclohexane): $\lambda_{\text{max}} = 219 \text{ nm}$ ($e = 1400$). $[\alpha]_{\text{D}} = +63^\circ$ ($c \approx 2 \text{ mg}$ in 1.0 ml CCl_4). EIMS m/z (rel. intensity): 220 (M, 7), 205 (24), 202 (58), 187 (42), 173 (16), 162 (82), 159 (48), 149 (31), 147 (46), 145 (74), 131 (63), 120 (64), 119 (70), 117 (30), 107 (65), 105 (100), 95 (29), 93 (63), 91 (91), 81 (34), 79 (62), 77 (47), 67 (29), 55 (43), 43 (88), 41 (74). EI-MS (peak C + Sylon-BTZ) m/z (rel. intensity): 292 (M, 3), 277 [M-15(CH_3)]. HRMS: calcd for $\text{C}_{15}\text{H}_{24}\text{O}$: 220.1827; found: 220.1809.

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EFFECTIVE ATTRACTION RADIUS: A Method for Comparing Species Attractants and Determining Densities of Flying Insects

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Abstract—The catches of bark beetles (Coleoptera: Scolytidae) were compared between attractive traps releasing semiochemicals and passive traps (cylindrical sticky screens hung, at 10 heights of 0.7–11.5 m, on poles). A central attractive-trap pole was surrounded by three passive-trap poles spaced 50 or 100 m away at the apices of an equilateral triangle. The catches of *Tomicus piniperda* and other scolytid species on the attractive-trap pole baited with host monoterpenes, or the catches of *Ips typographus* attracted to synthetic pheromone, were compared to passive trap catches in a Scots pine forest or in a Norway spruce clear-cut, respectively. Information about flight height distributions of the above scolytid species, and *Hylurgops palliatus*, *Cryphalus abietis*, *Pityogenes chalcographus*, *P. quadridens*, *P. bidentatus*, and *Trypodendron domesticum* were obtained on the passive and attractive trap poles. A new method is presented for determining the densities of flying insects based on the passive trap's dimensions and catch, duration of test, and speed of insect. Also, a novel concept, the effective attraction radius (EAR), is presented for comparing attractants of species, which is independent of insect density, locality, or duration of test. The EAR is obtained by the ratio of attractive and passive trap catches and the dimensions of the passive trap, and thus should correlate positively with the strength of the attractant and the distance of attraction. EARs are determined from catch data of *T. piniperda* and *I. typographus* as well as from the data of previous investigations on the same or other bark beetles.

Key Words—Bark beetle, *Ips typographus*, *Tomicus piniperda*, Coleoptera, Scolytidae, pheromone, host attractants, dispersal, flight, *Pityogenes*, *Hylurgops*, *Cryphalus*, *Trypodendron*

INTRODUCTION

It is of ecological interest to determine the relative abundance of flying insects at various heights above ground. Also, such knowledge should be considered when using pheromone traps at certain heights for monitoring the abundance of a pest species. Information concerning the density of flying insects (per volume air or per area over land) is often required to build models of host and/or mate location, dispersal, and disease vector relationships. It is also of interest in chemical ecology to be able to compare the attractivities of various attractants of different strengths or blends within a species as well as between species at different times or under different environmental conditions. For instance, one question might be whether host attractants are relatively as attractive to one species, which does not employ long-range pheromones [*Tomicus piniperda* (L.); Byers et al., 1985], as are pheromone components to another species [*Ips typographus* (L.); Schylter et al., 1987].

We present a method that provides information helpful for a better understanding of the above three topics: flight height, flight density, and comparison of attractant strengths both within and between species. The method involves comparing the catches of flying insects, exemplified by bark beetles, that are passively intercepted by cylindrical sticky screens suspended at various heights above ground on 12-m poles to the catches of these insects attracted to attractants released from sticky-screen traps on a similar pole in the same area.

METHODS AND MATERIALS

It is obvious that passive insect traps placed at various heights above ground will catch the proportion of insects flying at each of the respective heights. However, we believe such an arrangement of traps can also be used to determine with reasonable success the average densities of insects flying in the vicinity of the traps. Cylindrical sticky screens in the forest can be considered as "filtering" or intercepting bark beetles that fly or are carried through the area during their dispersal and host- and mate-seeking flight. If one had a cylindrical trap as long as a standing tree, or at least sample traps at many heights encompassing most of the levels of flight activity, then one can consider this system in a two-dimensional sense. The trap then can be visualized as a circle on a plane surface. The trap (or circle) then will catch an average number that is dependent on the trap's diameter ($2 \times$ radius), time length of trapping, average speed of flying beetles, and the density of flying beetles (number per area) as shown in equation 1:

$$\text{Catch} = 2 \times \text{radius} \times \text{time} \times \text{speed} \times \text{density} \quad (1)$$

However, if the passive sticky traps with a certain radius are placed in the forest for a specific time and catch, and we assume an average flight speed (either from observations of flying beetles or assuming a flight speed equal to the wind speed), then the density can be determined:

$$\text{Density} = \text{catch}/(2 \times \text{radius} \times \text{time} \times \text{speed}) \quad (2)$$

Passive and attractive (attractant releasing) traps can also be used in a new concept and method, the effective attraction radius (EAR), for comparing semi-quantitatively the attraction distances of attractants both within and between species of insects. A maximum possible distance of attraction can be imagined with the earlier concept of the active space, in which a time-averaged volume (plume) containing above-behavioral-threshold semiochemical concentrations elicits attraction responses when entered by the insect (Bossert and Wilson, 1963; Nakamura and Kawasaki, 1977; Baker and Roelofs, 1981; Elkinton and Cardé, 1984). This concept can be modified to an attraction space to find distances from the source within which, for example, 50% of the entering insects are successful in finding the source. The average distance of attraction is another measurement which may be of interest. We now introduce a new concept, the EAR, which is equivalent to the distance from an attractive source within which all insects are assumed to be attracted. The EAR is the radius of a circular plane oriented perpendicular to the incoming insects and thus can be regarded as the radius of a spherical volume that surrounds the attractive source:

$$\text{EAR} = (\text{ATC} \times \text{LCSAPT} \times \text{PTC}^{-1} \times \pi^{-1})^{1/2} \quad (3)$$

where ATC is the attractive trap catch, PTC is the passive trap catch, and LCSAPT is the longitudinal cross-sectional area of the passive trap.

It is probable that "all" insects are never attracted to semiochemicals if within a specific distance of the source, and thus the EAR is not a "real" biological distance. However, the EAR does have positive relationships to the "real" maximum, 50% responding, and average distances of attraction as mentioned in the concepts above. While the active space model, the attraction space model, or the average attraction distance cannot be investigated easily with trap catches (Elkinton and Cardé, 1984), the EAR can be found in a straightforward manner by simple comparison of two trap catches. For example, if a passive trap area in longitudinal cross section, A , containing no attractants, intercepts X number of beetles per time unit, and if, in the same vicinity, a similar trap containing attractant catches $20X$ beetles per time unit, then the effective area (longitudinal cross section) of the attractive trap is 20 times larger than the passive trap, and the $\text{EAR} = (20A/\pi)^{1/2}$. This ratio of catch between the passive and attractive traps should remain similar regardless of the density of flying beetles, so consistent measurements of the EAR can be obtained on different

dates or in different areas. The type and strength of attractant should be the most important factor in affecting the ratio.

To demonstrate our ideas concerning measurement of flight density and EAR, we placed metal poles holding cylindrical sticky screens at 10 levels (from 0.7 to 11.5 m) in or near the forest. Three poles with passive traps were positioned at the apices of an equilateral triangle while a fourth pole with attractive traps (host attractants or pheromone) was placed in the center of the triangle. Each pole was 12 m high and constructed of four 28-mm diameter steel tubes (3 m long) interconnected and held upright by two sets of four guy wires attached at 6- and 12-m heights. At the top of each pole was a 30-cm arm with pulley which suspended a string of 10 cylindrical sticky screens so that they could be drawn up or down the pole between test periods. Each sticky screen was 30 cm tall by 15 cm radius (LCSAPT of 0.09 m²) of 6.5-mm square mesh coated with Stikem Special (Seabright Enterprises, Emeryville, California).

The attraction of *Tomicus piniperda* (L.), the European pine shoot beetle, and associated bark beetles to host monoterpenes (Byers et al., 1985) was investigated using the poles inside a Scots pine (*Pinus sylvestris* L.) forest, 60 years old, near Ångelholm, Sweden (April–May 1984). The passive poles were 50 m from the central attractive pole, forming a triangle of 87 m on a side. Each tubular trap had two open polyethylene vials (No. 730 Kartell, Italy), 30 mm × 6-mm ID, for each of the host monoterpenes, (+)- α -pinene (99% pure by GLC; $[\alpha]_{546}^{20} = +57.4^\circ$), (-)- α -pinene (>99.5% GLC; $[\alpha]_{546}^{20} = -50 \pm 1^\circ$), (+)-3-carene (>99% GLC; $[\alpha]_{\text{D}}^{20} = 17 \pm 0.5^\circ$), and terpinolene (>97.3% GLC). Chemicals were from Fluka AG, West Germany, and Carl Roth, Sweden. Each trap released about 10–20 mg/day of each of the monoterpenes except for about 3–5 mg/day of terpinolene (these rates are equivalent to that released from a freshly cut Scots pine log, 28 cm × 15 cm diam.; Byers et al., 1985).

Flight characteristics of *Ips typographus* (L.), the European spruce bark beetle, were similarly studied within a year-old clear-cut area adjacent to Norway spruce [*Picea abies* (L.) Karst.] forest in Esrum forest district, near Hillerød, Denmark (May 1984). The passive poles were positioned 100 m from the central attractive pole in the corners of a triangle 173 m on a side. These distances between passive and attractive trapping poles were chosen because trap and semiochemical interactions are minimal (Schlyter et al., 1987; Byers, 1987) while population levels of flying beetles are still expected to be rather uniform within this area. Each cylindrical trap on the attractive pole had two pheromone dispensers, which released 5 mg/day of 2-methyl-3-buten-2-ol and 0.1 mg/day of (4*S*)-*cis*-verbenol (Schlyter et al., 1987). Trapping in both areas was done in the afternoon for the time periods and dates as shown in Table 1. Wind speeds were taken with a fan anemometer. Bark beetles of all species caught on the sticky traps during each period were removed at the end of the test time, cleaned in petroleum ether, and sex determined for *T. piniperda* and *I. typographus*.

Since it is possible that too small an active trap would catch a dispropor-

TABLE 1. NUMBER OF BARK BEETLES CAUGHT ON PASSIVE AND ACTIVE STICKY-SCREEN TRAPS, TRAPPING PERIODS, AND WIND SPEEDS USED TO CALCULATE EARS OF BAITS AND DENSITIES OF FLYING BEETLES^d

Date 1984	Beetles caught		Wind (m/sec)	Trapping duration (min)	EAR ^b (m)	Flying beetles/hectare estimated from passive trap poles			
	Passive traps	Active traps				A	B	C	Average
<i>Tomicus piniperda</i> ^c									
April 15	7	27	<0.5	240	0.58	17	6	17	13
April 21	3	19	<0.5	240	0.74	6	12	0	6
<i>Ips typographus</i> ^d									
May 17	17	194	2.23	150	0.99	93	13	7	38
May 19	4	18	4.00	160	0.62	14	0	0	5
May 20	50	269	2.70	250	0.68	128	30	7	55
May 21	15	44	3.76	235	0.41	(>58) ^e	30	8	(>32)
May 22	17	215	1.56	135	0.85		(148) ^f	32	90

^aDensities were calculated from equation 2 using trapping durations as shown and beetles were assumed to fly at the respective wind speeds (catch = actual catch × 4 to correct for incomplete trapping surfaces).

^bBased on average catch of passive trap poles.

^cDensities of flying beetles based on observed flight speed of 1.6 m/sec; attraction of beetles to host monoterpene mixture.

^dDensities of flying beetles based on wind speeds; attraction of beetles to pheromone components.

^ePole blown down during experiment.

^fBeetle density actually measured at center location, while pheromone bait was at position B.

tionately small number attracted to the source and thus adversely affect measurement of the EAR, we tested four trap sizes each with the same pheromone bait as above on *I. typographus*. Cylindrical sticky screens were similar to those above and of the same length (30 cm) but varied in radius, at 3.75, 7.5, 15, and 30 cm. These traps were each held at 1.5 m height by wires from a 22-mm-diam. steel tube driven into the ground. Two sets of each of the four sizes were tested in May and June of 1984 and 1985 for a total of 21 replicates with rotation among positions. Linear and logarithmic regressions of the total catches and of the proportions among trap sizes at each trap radius were compared.

RESULTS

Height above Ground of Flying Bark Beetles. Catches of bark beetles by the passive sticky screens indicate that most species fly above 0.7 m (the lowest trap level), are most abundant at 1.9–5.5 m, and gradually fly less frequently at still higher levels (Figures 1 and 2). This pattern is shown best by *Hylurgops palliatus* Gyll., *Pityogenes bidentatus* Hbst., *P. quadridens* Hart., *Trypoden-*

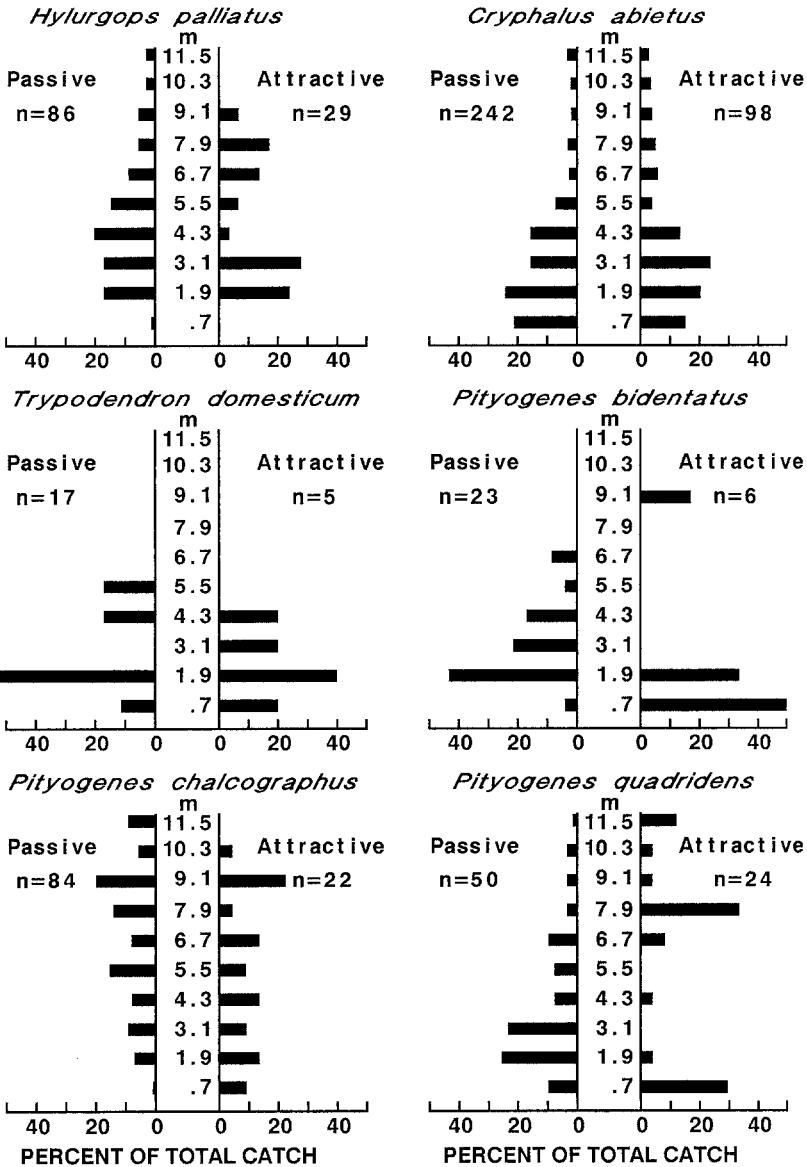


FIG. 1. Height of flying bark beetles as percent of total catch on passive sticky-screen traps or "attractive" sticky-screen traps releasing host monoterpenes at each level [(+)- α -pinene, (-)- α -pinene, (+)-3-carene each at 10-20 mg/day and 3-5 mg/day of terpinolene]. Passive traps at each height on three 12-m poles were spaced 50 m away from a central active trap pole in Scots pine forest (May 1, 1984).

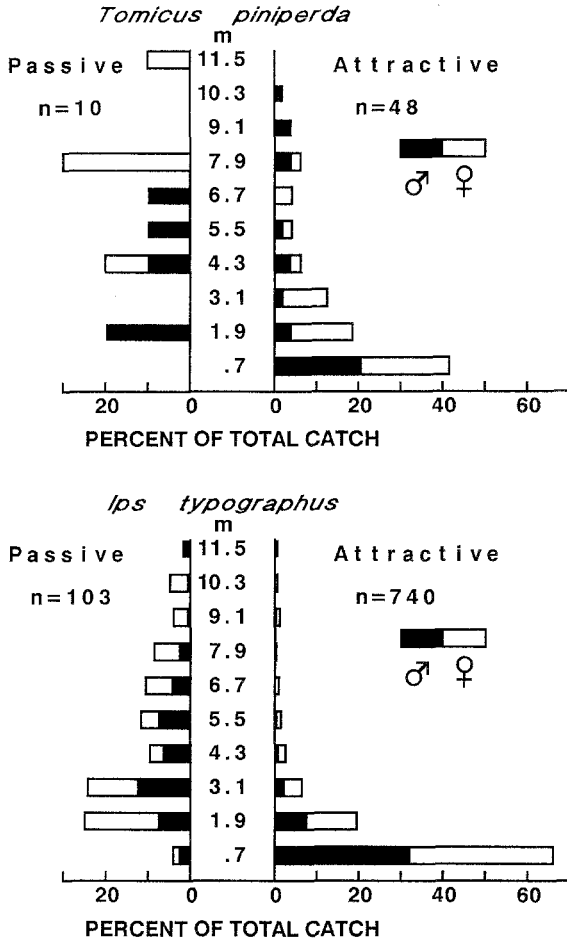


FIG. 2. Height of flying bark beetles as percent of total catch on passive sticky-screen traps or attractive sticky-screen traps releasing host monoterpene attractants (*T. piniperda*) or pheromone components (*I. typographus*). Placement of trap poles was as in Figure 1 for *T. piniperda* (April 15 and 21, 1984). For *I. typographus* the passive poles were spaced 100 m away from the attractive pole in a clear-cut of Norway spruce forest (May 17, 19, 20–22, 1984). Monoterpene attractant release as in Figure 1, while the pheromone components, 2-methyl-3-butene-2-ol and *cis*-verbenol, were released at 5 and 0.1 mg/day, respectively, at each trap level.

dron domesticum L. (although catch was low), and *I. typographus*. *P. chalcographus* L. and *T. piniperda* also did not fly near the ground but they seemed to exhibit a more uniform distribution of flight heights (Figures 1 and 2), although the numbers for *T. piniperda* are too low for reliable estimates.

Most of the distributions of beetles at the heights seemed unaffected by semiochemicals since chi-square comparisons of the passive and attractive distributions yielded no significant differences for each species ($P > 0.1$) except for *P. quadridens* ($P < 0.05$), *T. piniperda* ($P < 0.05$), and *I. typographus* ($P < 0.001$). However, the distributions of *P. quadridens* on the attractive traps is based on a low number, and it is difficult to explain the pattern. The pattern of *T. piniperda* is also based on low numbers, but it is consistent with the pattern for *I. typographus* in which attractive pheromone traps catch proportionately more beetles on the lowest traps (Figure 2).

Density of Flying Bark Beetles. The number of bark beetles caught on passive and attractive sticky-screen traps, trapping periods, and speeds of beetles (either observed or assumed to be equal to the measured wind speed) were used to calculate the densities of flying beetles per hectare in the level from 0 to 12 m using equation 2 and multiplying by 4 to account for the gaps between traps (Table 1). The densities of flying beetles (per hectare) were estimated for each trap pole on each day for *T. piniperda* and *I. typographus* (Table 1) and for six other species of bark beetle (Table 2) to indicate the variation in density with respect to time, area of forest, and species.

TABLE 2. DENSITIES OF FLYING BARK BEETLES IN SCOTS PINE FOREST NEAR ÄNGELHOLM, SWEDEN, DURING AFTERNOONS (1330–1730 Hr) OF SPRING DAYS AS ESTIMATED FROM PASSIVE STICKY SCREENS ON 12-M POLES^a

Species	Flying beetles per hectare estimated from trap poles			
	A	B	C	Average
April 15, 1984				
<i>Hylurgops palliatus</i>	52	179	81	104
<i>Trypodendron domesticum</i>	6	29	17	17
April 21, 1984				
<i>Hylurgops palliatus</i>	6	29	98	44
<i>Trypodendron domesticum</i>	6	6	12	8
May 1, 1984				
<i>Hylurgops palliatus</i>	12	23	17	17
<i>Trypodendron domesticum</i>	6	12	6	8
<i>Pityogenes chalcographus</i>	174	87	226	162
<i>Pityogenes quadridens</i>	116	81	93	97
<i>Pityogenes bidentatus</i>	52	75	6	44
<i>Cryphalus abietis</i>	307	729	365	467

^aDensities were calculated from equation 2, where trapping was for 4 hr; all species were assumed to fly at 1.6 m/sec as observed for *T. piniperda*; and catch = actual catch \times 4 to correct for incomplete trapping surfaces.

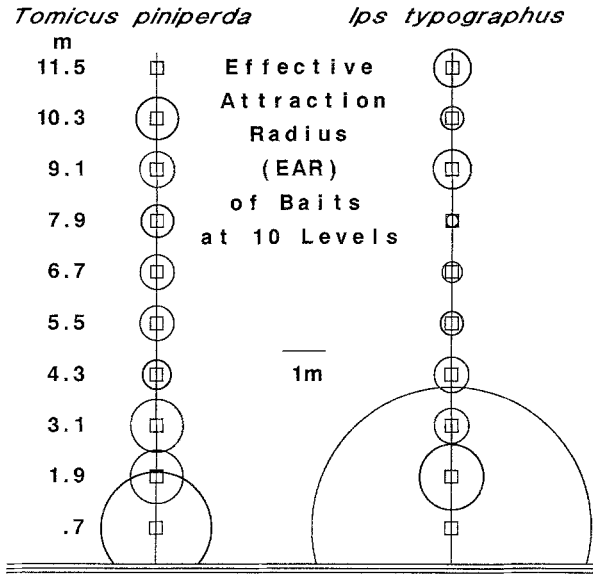


FIG. 3. Comparisons of the effective attraction radii (EAR) of attractive, monoterpene-releasing, sticky-screen traps (*T. piniperda*) or of pheromone-releasing, sticky-screen traps (*I. typographus*). Release of semiochemicals as in Figures 1 and 2.

Effective Attraction Radius for Comparing Attractants of Insects. The average of EARs for the traps on the entire trap pole in both *T. piniperda* and *I. typographus* were similar as were the EAR estimates for each day (Table 1). The lowest EARs for *I. typographus* were correlated with the highest wind speeds (Table 1). The EAR was largest at the bottom trap level for both species, and it was larger for the pheromone compared to host monoterpenes at the dosages used (Figure 3). At other levels, the EARs were similar within a species as well as between the two species. The sticky-screen traps of increasing radius (3.75–30 cm) caught increasing numbers of beetles in a logarithmic relationship (Figure 4). This curve may be expected if beetles have increasing difficulty finding a point source of pheromone (i.e., must turn more rapidly than possible near the source). The coefficient of determination ($r^2 = 0.90$) was larger than that for a linear relationship ($r^2 = 0.90$).

DISCUSSION

Height above Ground of Flying Bark Beetles. Several studies have investigated the height at which a particular species of bark beetle flies. However, most of these studies have used only: (1) traps with pheromone, (2) traps at just

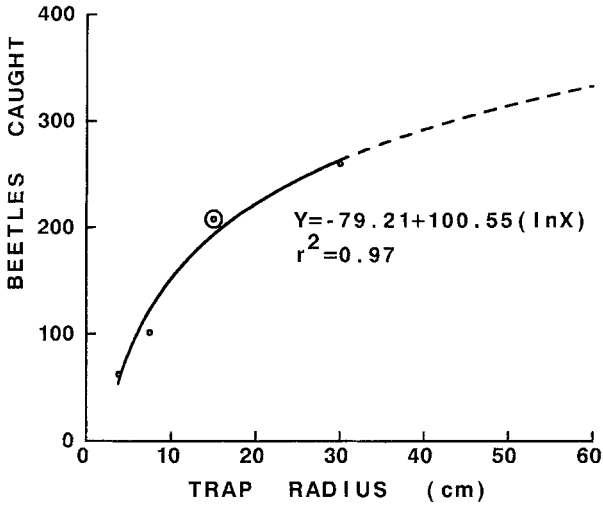


FIG. 4. Logarithmic relationship between number of *I. typographus* caught on pheromone baits and increasing radius of the sticky-screen trap at 1.5-m height. Traps released 5 mg/day of 2-methyl-3-butene-2-ol and 0.1 mg/day of *cis*-verbenol. The encircled point shows the size of trap used in the attractive and passive pole experiments.

a few levels, (3) traps at widely spaced levels (up to 150 m height), or (4) traps that collected beetles for several months. Our 10 passive traps from 0.7 to 11.5 m high appeared sufficient to determine with some precision the distribution of flight heights over the normal flight range for *H. palliatus*, *Cryphalus abietis* Ratz., *T. domesticum*, *P. bidentatus*, *P. quadridens*, and *I. typographus* but not, apparently, for *P. chalcographus* and *T. piniperda* (Figures 1 and 2). The catch for *T. piniperda* was too low to reliably estimate the distribution. On the other hand, *P. chalcographus* exhibited a uniform distribution, except at the bottom level, which probably indicates they were flying also at higher levels than the range of traps, as if they were dispersing through the pine forest (a nonhost tree).

In one of the first studies of flight-height distribution in bark beetles, Gara and Vité (1962) used rotary net traps to suggest that no differences in flight activity of *Dendroctonus brevicomis* LeConte and other bark beetles occurred from ground level to lower crown regions, but "in the upper crown and above forest stands, bark beetles seemed to fly less frequently." Shore and McLean (1984) found *Trypodendron lineatum* Ol. responded over 76 days to lineatin-baited traps at 0-, 1-, 2-, 3-, and 4-m heights with most (31.6%) caught on the 1-m trap at the level just above the underbrush (effectively the lowest trap). The

highest catch on the "lowest" trap of a column of traps releasing semiochemicals is the same result we found for *T. piniperda* and *I. typographus* (Figure 2). It is possible that these high catches on the lowest trap were due either (1) to beetles flying lower in order to avoid flying against higher wind speeds, (2) to increasing inability to orient to the source at higher levels due to relatively stronger, more turbulent wind or a decrease in the optomotor response further from the ground, or (3) to the absence of a visual silhouette (tree) in conjunction with odor stimuli, and thus they were trying to land on a fallen tree.

Schmitz (1980) intercepted *I. pini* Say as they flew to a source of naturally produced pheromone (0- to 2-m height) by four columns, one in each cardinal direction, of 11 flat sticky traps from 2 to 17 m high about 45 m from the source. He found most beetles flew below 10 m as they approached the source. However, these results cannot be compared either to our passive trap catches or to our column of semiochemical release.

Cuthbert and Peacock (1975) placed flat sticky traps baited either with multilure or not at about 3.5-, 7-, and 12-m heights in healthy or diseased elm trees. They found that *Scolytus multistriatus* Marsh. was caught predominantly at the 3.5-m height in baited or unbaited traps, a result similar to our studies. Forsse and Solbreck (1985) placed suction traps at 2, 9, 43, and 93 m on a TV tower and caught *I. typographus* only at 2 and 9 m. They report that the density-height profile of all bark beetle species caught (Solbreck, 1985) indicates that less than 14% of any species flies above 20 m. Using a similar regression technique (exponential without 0.7-m height for all $r^2 > 0.78$), we predict that 87% of *I. typographus* fly between 1 and 11.5 m high of all those flying up to 100 m high. Similarly, 84% of *H. palliatus*, 89% of *C. abietis*, and 89% of *P. quadridens* fly below 11.5-m height.

Duelli et al. (1986) placed pheromone traps near passive traps at nine heights from 1.7 to 150 m on a weather tower and found that less than 5% of *I. typographus* fly above 10 m. In contrast, *P. chalcographus* had a more gradual decrease in proportion, flying at higher levels (14% flew higher than 10 m), which is similar to our results for this species (Figure 1). However, their results on the passive catches are uncertain because complete or partial pheromone blends were placed at the same heights on the tower as were the passive traps.

Density of Flying Bark Beetles. The density of flying insects can be determined either by active methods, whereby suction fans or rotary nets capture insects, or by passive methods such as our sticky traps. Our investigation is the first to use such passive methods to determine densities of flying beetles, but the results of previous studies can be reanalyzed according to equation 2 for comparison. However, studies that report catches of bark beetles in barrier traps are less accurate for this purpose because beetles may avoid them due to air turbulences or may recover their balance after striking the barrier (Chapman

and Kinghorn, 1958). Also, a conversion from the width of flat traps to a diameter ($2 \times$ radius) for tubular traps must be done according to the average trap interception area:

$$2 \times \text{radius} = 2 \times \text{width} * \pi^{-1} \int \cos A = 0.637 \times \text{width}$$

Thus, assuming a 5-hr flight for each day of a 37-day test and 2 m/sec speed of beetle or wind, then Cuthbert and Peacock (1975) had a density of flying *S. multistriatus* in the healthy elms (0–12 m height) of 1/hectare and in the diseased elms of 2069/hectare. The results of Schmitz (1980) for *I. pini* can be similarly adjusted to yield an average density of 43/hectare (0- to 17-m height) at 45 m away from a source of pheromone. Byers et al. (1985) report a catch of 52 *T. piniperda* on two blank sticky traps (25 cm diam.) for 15 hr (time unpublished) during the maximum swarming, which yields about 401 flying beetles per hectare (0–10 m), clearly a higher population level than that in Table 1.

Our method of flight density estimation assumes that beetles do not avoid, or are attracted to, the passive traps and thus cause us to under- or overestimate the population level. Nijholt (1983) questioned whether sticky-screen traps appear transparent to bark beetles because he found a nonrandom distribution of catch on a 60×80 -cm-wide screen held 60 cm above ground. The average catch of the peripheral areas of the trap was less than the center. However, it is possible that through the beetles' movements to free themselves from the stickem, they gradually slid down the sticky screens so that the top periphery of the trap was depleted of beetles over time, thus accounting for the difference. Furthermore, the bottom area may have caught less beetles simply because fewer beetles fly near the ground.

Bark beetles are known to respond to visual silhouettes of the size of a tree trunk often in combination with attractive semiochemicals (Tilden et al., 1983; Borden et al., 1986; Payne, 1986). The resolving power of insect eyes depends on the number of facets and the ommatidial angle. Insects with higher visual acuities have higher numbers of ommatidia; the eye of the lamellicorn beetle *Polyphylla fullo* has 12,150 facets, the housefly about 4000, and dragonflies about 10,000–28,000 (Wigglesworth, 1972). Bark beetles are known to have rather low acuity, which is explained by the low number of ommatidia. Using scanning electron micrographs of several genera of Scolytidae, we found that the eyes of all species have similar low numbers of ommatidia. For example, *I. typographus* has 215 facets per eye, *T. piniperda* also has 215, *Scolytus laevis* Chap. has 235, and *P. chalcogaphus* only 110. In comparison, the bark beetle predator, *Thanasimus formicarius* (L.) (Cleridae), has 610 facets per eye, and the cerambycid, *Acanthocinus aedilis* (L.), has about 650.

Our sticky-screen traps were at least 75% transparent while Stikem Special

is translucent, thus, the traps are similar to spider webs, which are effective in catching insects with moderate to poor visual acuity. Therefore, it is probable that the bark beetle species collected neither were attracted to nor avoided the visual outlines of the sticky traps.

As mentioned above, capture methods have usually been used to estimate flight densities. Chapman and Kinghorn (1958) used 6 m/sec rotary nets 2 m above log piles during spring swarming of *T. lineatum* to find maximum densities of 1.521/m³. This value can be converted to densities for a 0- to 12-m layer (182,520/hectare) or for a 0- to 3-m layer (45,630/hectare). Forse and Solbreck (1985) used suction traps that generated an airflow of 1.67 m³/sec to capture bark beetles at four heights. However, estimation of densities from this method is doubtful as insects are only captured when very near the suction inlet. This distance of capture is hard to estimate because it is different for every insect, depending on their size and speed (Leos-Martinez et al., 1986).

The results of our passive sticky-screen catches indicate that at the densities we measured, few trees in the forest would escape being closely passed by or landed upon by the common bark beetle species. This is in accordance with findings for *D. ponderosae* Hopkins (Burnell, 1977; Raffa and Berryman, 1980; Hynum and Berryman, 1980), showing that beetles land on most trees in the forest and that this pattern can result from randomly flying beetles. Primary attraction indicating more susceptible hosts to *T. piniperda* (Byers et al., 1985) would thus not need to reach very far (on the order of our EARs) to attract the pioneer beetles. If the host conditions were suitable, these pioneers could generate aggregation pheromone (although this does not appear to be the case for *T. piniperda*) or increase the release of the primary attractants through boring activities (secondary attraction).

Effective Attraction Radius for Comparing Attractants of Insects. Vité and Gara (1962) hypothesized that the number of bark beetle attacks in a source of attractant influenced the distance over which beetles were drawn, but few experiments have tested this or determined the distance-concentration relationship. Using rotary-net traps at the pheromone source and at 15 and 30 m away, Vité and Gara (1962) showed that *I. paraconfusus* Lanier were attracted from at least 15 m away to the infested logs. Possibly even at 30 m there was an increased capture rate, but control data were not presented to establish a difference. Byers (1983) used a grid of passive sticky traps in three rows distant from a pheromone source of *I. paraconfusus* to show that "wild" beetles of both sexes were attracted from at least as far away as 18 m. While an average or a maximum attraction distance must exist for each specific attractant/species combination, they are difficult to determine, as noted above. On the other hand, the EAR is easy to establish and yields an index of the attraction distance so that comparisons can be made between different times, areas, semiochemical releases, and insect species.

EARs were calculated at 10 heights for *T. piniperda* attraction to monoterpenes released equivalent to a "small log" (28 cm × 13 cm diam.; Byers et al., 1985) and for *I. typographus* attraction to "medium" levels (Schlyter et al., 1987) of pheromone (Figure 3). However, these estimates at different heights may have been greatly influenced by semiochemical interactions between traps. It would be interesting to test only one pair of traps alone at each of the levels to see how this affects the EARs. The EAR estimates for *T. piniperda* appear similar to those that can be obtained from the data of Byers et al. (1985) for a small log (EAR = 0.59 m) or a 30 male + 30 female infested log (EAR = 0.65 m) or small log-equivalent in monoterpene release (EAR = 0.91 m) and check log (EAR = 1.02 m) at 1.5 m height.

Our EAR estimates for *I. typographus* were probably affected by the higher than usual wind speeds in the clear-cut (Figure 3, Table 1). Schlyter et al. (1987) compared a "high" dose (10 × "medium") of *I. typographus* pheromone to a blank, 12 m away at 1.5-m height, and so we can calculate an EAR of 1.90 m, and for their "medium" dose an EAR of 0.57 m. If the blank had been placed further away, so as not to intercept any pheromone-responding beetles, then the EAR estimate would have been higher and more appropriate. Thus, as expected, higher release rates of pheromone have higher EARs. Similar procedures can be used to calculate EARs on previous work that used sticky traps. For example, Byrne et al. (1974) used sticky traps to catch 6724 *Gnathotrichus sulcatus* on two sulcatol-baited traps (each releasing 48–240 µg/day) versus 51 on two control traps (0.0954 m² interception area), which yields an EAR of 2.00 m.

An EAR can be calculated for any semiochemical and responding insect species if the passive trap effectively "filters" the flying population according to the trap's size (sticky traps). However, the attractive trap, while not needing to be of the same type or dimension as the passive trap, does need to (1) effectively catch a significant proportion of the insects responding and (2) be similar or smaller in size than the passive trap in order to obtain consistent EARs for a particular chemical–insect combination. The trapping efficiencies (percentage of beetles contacting the trap that are caught) of the passive and attractive traps within a test pair would not affect the EAR, as long as the efficiencies were similar. The efficiencies could even be different on different occasions and not change the EAR unless the efficiencies change disproportionately between the passive and attractive traps.

Trap size does effect trapping efficiency since Tilden et al. (1979) showed that many more *D. brevicornis* were attracted to the vicinity of a pheromone source and small surrounding trap than were caught on this trap. EAR estimates obtained by increasing the passive and attractive trap radius would tend to increase as a function of the trapping efficiency (size) of the attractive trap

(Figure 4). Thus, the question arises: how does one determine the optimal size of attractive trap to obtain consistent and maximum EAR estimates?

Ideally, one should compare several attractive traps of increasing radii to determine the function of trapping efficiency with trap size and the effect on the EAR. In this way, the appropriate trap size can be determined that yields a consistent and nearly maximum EAR, with a minimum of trap size for practical reasons. In *I. typographus* (Figure 4), we found that little further benefit in maximizing the EAR estimate is gained with increasingly larger attractive traps beyond 15- to 30-cm radius (we used the 15-cm radius on the poles), while there are increasing costs of further enlargements of trap radius. It can also be seen in Figure 4 that *I. typographus* seem to find it increasingly more difficult to locate pheromone sources that are much smaller than the diameter of a tree. We predict that such curves for moth species will be shifted to the left on the x axis, indicative of the smaller target of a female moth.

Several environmental factors will influence the active space (Elkinton and Cardé, 1984), as well as the EAR, such as temperature, wind speed, and their variation over time. However, these parameters are rather consistent when bark beetles are responding to semiochemicals. For valid EAR measurements, the height of the passive and attractive traps should be the same but the level is, in theory, not too important as different heights would appear as tests at different densities of flying beetles, which has little effect on the EAR. However, our measured EARs at different heights indicate that height may be important either due to windy conditions, response variation with height, or trap interactions.

Thus, it appears that EAR estimates for a particular species should be qualified by specifying the height of the attractive and passive traps, the release rate of the semiochemical, and the sizes of the traps. Since most pheromone catches of scolytids have been done at the 1.5-m height, and we obtained the largest EAR at about this level, we think this level is appropriate for interspecific comparisons.

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COLLECTION OF PHEROMONE FROM ATMOSPHERE
SURROUNDING BOLL WEEVILS,
Anthonomus grandis^{1,2}

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Abstract—An effluvial method was developed to collect the pheromone, glandure from actively calling male boll weevils, *Anthonomus grandis* Boheman. The adsorbant, Porapak Q (ethylvinylbenzene–divinylbenzene), was utilized to trap and concentrate the pheromone. Captured pheromone was desorbed from columns packed with Porapak Q by elution with *n*-pentane and quantified by capillary column gas–liquid chromatography. In recovery studies with known amounts of synthetic glandure, we found that the amount of each pheromone component collected was a function of collection duration, elution volume, and initial concentration. This effluvial method was capable of recovering as much as 94.9% of a known quantity (80 µg) of glandure. The chromatograms were free of extraneous peaks. In studies of insect-produced pheromone, the effluvial method was used to collect pheromone from the air space surrounding male boll weevils as they fed on flower buds from CAMD-E cotton. The quantity and quality of boll-weevil-produced pheromone was determined for days 6, 8, 10, 11, 12, 13, and 14 of boll weevil adulthood. The maximum quantity of natural pheromone was produced on day 13 (4.2 µg/weevil) with a pheromone component ratio of 2.41:2.29:0.95:1 for components I, II, III, and IV, respectively. The effluvial method described in this report is an efficient method to collect and quantify boll weevil pheromone from the atmosphere surrounding actively calling insects. Other applications of this method are suggested.

¹Coleoptera: Curculionidae.

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Key Words—*Anthonomus grandis*, behavior, boll weevil, Coleoptera, cotton, cultivar, Curculionidae, effluvial method, grandlure, pheromone, Porapak Q, pheromone collection, aeration collection.

INTRODUCTION

The ability of boll weevils, *Anthonomus grandis* Boheman, to locate the host plant, *Gossypium hirsutum* L., is thought to be mediated, at least in part, by the insect's olfactory response to host-plant volatiles and the multicomponent pheromone, grandlure. The pheromone is produced by males as they feed on pollen in flower buds. Boll weevil pheromone composition has been determined from the frass by means of steam distillation (Tumlinson et al. 1969). The pheromone consisted of four terpenoid components, two alcohols [I, (+)-*cis*-2-isopropenyl-1-methylcyclobutane ethanol; and II, *cis*-3,3-dimethyl- $\Delta^{1-\beta}$ -cyclohexane ethanol] and two aldehydes [III, *cis*-3,3-dimethyl- $\Delta^{1-\alpha}$ -cyclohexane acetaldehyde; and IV, *trans*-3,3-dimethyl- $\Delta^{1-\alpha}$ -cyclohexane acetaldehyde], in the ratio 12:9:1:1, respectively. For the pheromone to stimulate an attractive response in boll weevils, both alcohol components and at least one of the aldehydes must be present (Tumlinson et al., 1969). The pheromone acts as an aggregant by itself (Lopez et al., 1978) and becomes a sex attractant in the presence of cotton-plant volatiles (Dickerson et al., 1981; Hardee et al., 1969). However, the actual composition of pheromone in the atmosphere surrounding actively calling males has not been determined. Collection of boll weevil pheromone from the atmosphere surrounding actively calling boll weevils would provide accurate quantification of the naturally released pheromone components and their ratios.

Porapak Q has been used to trap and concentrate bark beetle host volatiles (Pearce et al., 1975) and a blend of bark beetle pheromones from actively calling insects (Peacock et al., 1975). However, the effectiveness of Porapak Q as a trapping agent was reported to be limited by its low thermal stability and the production of artifacts in chromatograms from pheromone analysis (Golub and Weatherston, 1984; Zlatkis et al., 1973). In previous studies we have found that Porapak Q was an effective effluvial collection agent for airborne monoterpene volatiles, such as α -pinene, β -pinene, β -myrcene, *d*-limonene, and β -ocimene (Chang et al., 1986). Under our experimental conditions, no artifacts due to Porapak Q breakdown were encountered, suggesting that Porapak Q may be suitable for boll weevil pheromone collection.

In the present study we report on: (1) the efficiency of Porapak Q to trap and concentrate known quantities of the synthetic boll weevil pheromone, grandlure; (2) a method to collect natural pheromone from the atmosphere surrounding male boll weevils, as they actively feed and release pheromone; and

(3) the quantities and ratios of each pheromone component produced by boll weevils on days 6–14 of adulthood.

METHODS AND MATERIALS

Conditioning Porapak Q. Before Porapak Q (ethylvinylbenzene–divinylbenzene) was used for collection of pheromone, it had to be conditioned by heating at 180°C for 24 hr with a nitrogen flow of 30 ml/min, as described by Zlatkis et al. (1973). After conditioning, the Porapak Q was washed three times, 30 min each time, with dichloromethane (CH_2Cl_2) using a power stirrer to remove the residual monomers. Following the last wash, the dichloromethane slurry was poured into a 20 × 300-mm glass column with a medium fritted glass filter. Nitrogen was forced through the column at the rate of 1.8 liters of air per minute (LPM) for 1.5 hr to dry the polymer. The solvent effluent from the column was drawn into a vacuum trap using a Chapman-type filter pump. The Porapak Q was then utilized in the assembly of air-cleaning columns (to clean the air entering into the effluvial system) and pheromone collection columns.

Preparation and Activation of Air-Cleaning and Collection Columns. Columns were made of Pyrex glass tubing, 210 mm × 8 mm outer diameter (OD). Each air-cleaning and collection column was packed with ca. 1.0 and 0.6 g of Porapak Q, respectively. The Porapak Q was secured within the glass tubing between two acetone-rinsed glass wool plugs.

Prior to pheromone collection, the columns were cleaned and activated. To accomplish this, columns were heated and maintained at 120°C, while 8 cc of glass-distilled acetone were forced through each column, in 2 cc volumes, with a metal-tipped glass syringe (a 40 mm length of 8-mm-ID Teflon tubing was slipped onto the metal tip of the syringe and connected to the columns). This treatment removed water and water-soluble contaminants. The remaining acetone was removed by flushing with 1.8 LPM of nitrogen for 2 min. Each column was then reactivated by pushing 8 cc of CH_2Cl_2 , in 2 cc volumes through the column, then flushing and drying with 2.7 LPM of nitrogen for 4 min, and sealing with glass caps until utilized to capture pheromone.

Effluvial Method for Collection of Airborne Pheromone. The volatile pheromone collection apparatus consisted of 10 trapping units operated simultaneously by means of a manifold which was connected to a Gast rotatory vacuum pump (AS55NXGTE-4145). A single volatile collection unit consisted of a 473-ml boll weevil glass holding jar (Mason-type jar), with an inlet for the air-cleaning column and an outlet for the collection column. The columns were fit with two lengths of 8-mm-ID Teflon tubing inserted through the metal lid of the jar. A short length of tubing (30 mm) joined each air-cleaning column to

the jar. The outer end of the second piece of tubing (300 mm) was connected to a pheromone collecting column while the inner end extended to the bottom of the jar and was plugged with acetone-rinsed glass wool to keep the boll weevils from escaping. The jar lids were sealed with Teflon tape before the retaining ring was placed in position. Each collection unit was connected by Tygon tubing to a flowmeter (Matheson 7262). The air-flow rate through the system was maintained at 0.225 LAPM with the flowmeter. The air-flow path was from the atmosphere through the air-cleaning column, boll-weevil-holding jar, collection column, flowmeter, manifold, and vacuum pump (operated at 635 mm Hg).

Recovery of Grandlure with Effluvial Method. The capacity of Porapak Q to trap and concentrate grandlure was tested using two concentrations of grandlure, 10 and 100 $\mu\text{g}/\text{ml}$ per component. The grandlure concentrations were prepared from 99% pure grandlure components (Albany International, New York). Aliquots (2 ml) of each concentration (20 and 200 $\mu\text{g}/\text{component}/\text{aliquot}$) were pipetted separately into jars of the effluvial collection system, and each treatment was replicated eight times. Then the grandlure was trapped in the collection columns for 4 hr. The trapped grandlure from the collection column was eluted with 3 ml of HPLC grade *n*-pentane, and the volume of the eluate brought back to the initial 2 ml amount with *n*-pentane. α -Terpineol was used as the internal standard for capillary column gas-liquid chromatography (GLC) to adjust volume and retention time of pheromone components. Also the 20 μg of each grandlure component was also recovered at two different time intervals (4 or 8 hr), two different volumes of elution (2 or 3 ml), and quantified without α -terpineol. The recovery efficiency for this study was determined directly from the analysis of trapped grandlure from the standard concentrations.

Collection of Boll Weevil Pheromone with Effluvial Method. Boll weevils used in these studies were cultured by the USDA, ARS, Boll Weevil Research Facility, Starkville, Mississippi. They were reared to adulthood on artificial diet. Three cohorts of 200 newly emerged male boll weevils were kept in clear plastic containers (90 \times 170 \times 300 mm) with an organdy-covered opening 40 \times 120 mm at each end. The plastic containers were held in Percival environmental chambers at ca. 30°C and 16:8 hr photophase-scotophase. Initially a layer of debracted flower buds was placed in half of the container, which was positioned toward the light source (20 W cool white GE fluorescent lamp) of the incubator. The next day, a layer of fresh flower buds was placed in the empty second half of the container and turned towards the light source. After 48 hr, the old flower buds were removed and fresh ones added, placing the newly changed side toward the light source. This reduced the handling of the boll weevils during flower bud changes. After five days of feeding on the flower buds, a cohort of 100 individuals was drawn at random from each of the three

plastic containers and enclosed separately with 50 debracted flower buds in three glass jars of the effluvial collection system. The flower buds were freshly collected from CAMD-E cotton, cultivated at College Station, Texas, under normal agronomic practices. Each glass jar with 50 debracted flower buds and 100 male boll weevils was considered a replicate. Three replications were conducted for each collection day. Boll weevil pheromone was collected from 0800 to 1600 hr (CST) on days 6, 8, 10, 11, 12, 13, and 14 of adulthood.

Quantification of Natural and Synthetic Pheromone. The captured synthetic or natural pheromone was eluted from the collection columns with HPLC grade *n*-pentane into 1-dram vials by solvent gravity flow. The vials were sealed with Teflon-lined screw caps and stored at -45°C . When elution was completed, the remaining solvent was forced from the column with nitrogen.

Eluted pheromone samples were analyzed by GLC. Quantification of the pheromone was determined by injection $1\ \mu\text{l}$ of the eluate, with an on-column SGE syringe, into a temperature-programmed Tracor model 565 gas chromatograph equipped with an Alltech RSL-150 (30 mm \times 0.25 mm bonded FSOT) capillary column, SGE OC-3 on column injector, and flame ionization detector. The column was preheated at 60°C for 4 min and programmed to increase at a rate of $10^{\circ}/\text{min}$, until it reached 180°C , then maintained at this maximum temperature for 7 min. Throughout the analytical determinations the following conditions were maintained: injector temperature, ambient; detector temperature, 220°C ; hydrogen flow rate per minute (FRPM), 40 ml; air FRPM, 400 ml; nitrogen as carrier gas FRPM, 2.2 ml; and nitrogen as make-up gas FRPM, 21.4 ml.

The recovery studies were conducted using synthetic pheromone. Quantification of synthetic pheromone was determined by response factors from $1\text{-}\mu\text{l}$ injections of a standard containing $10\ \mu\text{g}/\text{ml}$ of each grandlure component and $10\ \mu\text{g}/\text{ml}$ of α -terpineol, the internal standard. The analog signals from the electrometer (detector) were processed by a Hewlett-Packard 3392A integrator for visual display of chromatographic peaks, retention times, peak areas, and calculation of the concentration of each grandlure component and α -terpineol. The internal standard reference was turned off at the integrator level when analyzing samples without α -terpineol. Recovery results for samples with and without α -terpineol served to compare the accuracy and repeatability of the recovered grandlure aliquots injected into the GLC for analysis. During pheromone collection, when no internal standard was used, three initial and one final injections were conducted daily with a standard containing $10\ \mu\text{g}/\text{g}$ grandlure component, to monitor the condition of the capillary column, based on retention time and shifts in calibration parameters.

All pentane eluates from the synthetic pheromone recovery studies were brought back to the initial 2-ml volume. The $200\ \mu\text{g}/\text{component}$ eluates were further diluted to 1:10. The individual grandlure components in the pentane

eluates from the collection columns were identified by comparing their retention times against the retention time of the 10- μg grandlure standard. To clearly identify the natural pheromone components in the samples collected from the air space surrounding actively calling boll weevils, a known dilution of the standard containing 1 μg /grandlure component was used to spike the pheromone samples collected from boll weevils.

RESULTS AND DISCUSSION

Recovery of Synthetic Pheromone with Effluvial Method. The effluvial method efficiently captured and concentrated synthetic grandlure, allowing a maximum recovery of 94.9% of a known amount (Table 1). The capacity of Porapak Q to capture known quantities of grandlure was mainly affected by the initial concentration, collection time, and volume of eluting solvent. A significantly higher percentage of grandlure was recovered from the treatment with 20 μg /component than the treatment with 200 μg /component, 93.7 and 85.2%, respectively (with an internal standard, a 4-hr collection period, and 3 ml of eluting solvent). A significantly higher percentage of grandlure was recovered using 3 ml of *n*-pentane than 2 ml, 93 and 86.9%, respectively (using an 8-hr collection period, 20 μg /component, and without an internal standard). The lower percent recovery with 200 μg suggests that some grandlure may have been lost because it passed completely through the collection column (hereafter called breakthrough). Breakthrough at 200 μg could be the result of the sudden release of a large volume of volatile material in the collection jar. Additionally breakthrough of grandlure may be due to the presence of the hexane solvent used to dilute the grandlure components, which was not present in the studies with natural pheromone. Breakthrough was not a concern with our studies of naturally released pheromone since the release of pheromone into the collection jar was gradual over an 8-hr period. Furthermore, two collection columns were placed in series in the same vacuum line during the collection of natural pheromone. We did not find pheromone breakthrough; all pheromone was captured in the first collection column.

Extraneous peaks on chromatograms have been reported in the literature by other researchers when Porapak Q was used (Byrne et al., 1975; Golub and Weatherston, 1984; Krumperman, 1972). They suggested that the extraneous peaks were breakdown products from the Porapak Q and/or breakdown products from the trapped compounds. We did not find extraneous peaks in the chromatograms, thus indicating that Porapak Q was not breaking down, and furthermore, it may have prevented degradation of the trapped grandlure components. This could be due in part to our experimental conditions and procedures for conditioning and activating of Porapak Q. We used solvents as

TABLE 1. PERCENT RECOVERY OF GRANDLURE WITH PORAPAK Q AS AFFECTED BY GRANDLURE CONCENTRATION, USE OF INTERNAL STANDARD, LENGTH OF COLLECTION PERIOD, AND VOLUME OF ELUTING SOLVENT

Int. Std.	Treatments ^a			Recovery of grandlure components (%) ^b				Total grandlure (%)
	Hr	ml	$\mu\text{g/ml}$	I	II	III	IV	
Present	4	3	10	92.1a	90.1a	96.5a	95.9a	93.4a
Present	4	3	100	83.4c	82.7b	87.2c	87.6b	85.2b
Absent	4	3	10	94.8a	92.6a	95.9a	96.3a	94.9a
Absent	4	2	10	88.7ab	88.4a	94.4ab	94.2a	91.4a
Absent	8	3	10	93.2a	89.9a	94.4ab	94.3a	93.0a
Absent	8	2	10	85.5bc	82.0b	90.7bc	89.4b	86.9b

^aInt. St. = α -terpineol, Hr = 4- or 8-hr periods, ml = 2 or 3 ml of *n*-pentane, and $\mu\text{g/ml}$ = 10 or 100 $\mu\text{g/ml}$ for each grandlure component.

^bThe grandlure components are: I = (+)-*cis*-2-isopropenyl-1-methylcyclobutane ethanol; II = *cis*-3,3-dimethyl- $\Delta^{1,\beta}$ -cyclohexane ethanol; III = *cis*-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde; IV = *trans*-3,3-dimethyl- $\Delta^{1,\alpha}$ -cyclohexane acetaldehyde. Means within columns followed by different letters are significantly different ($P < 0.05$, Duncan's multiple-range test).

cleaning and activating agents of Porapak Q at 120°C in a procedure requiring approximately 20 min. This procedure reduced the 24-hr heating periods, the concomitant nitrogen flushing, the special equipment, and the disassembly of the collection columns required by other researchers' procedures (Golub and Weatherston, 1984). The effluvial method described here is a practical and efficient method to capture and quantify grandlure in the atmosphere.

Collection of Natural Boll Weevil Pheromone with Effluvial Method. The mean total quantity of pheromone produced by the laboratory strain of boll weevils was dependent upon the age of the insect (Table 2). The largest amounts of pheromone released during the first two weeks of adulthood occurred from days 11 to 14, with a peak on day 13 (4.2 $\mu\text{g}/\text{insect}$).

The mean amount of each pheromone component is dynamic in that it changed with boll weevil age (Table 2). Using the concentration of component IV as the unit reference value, the ratios of the other three pheromone components were calculated for days 6, 8, 10, 11, 12, 13, and 14 of adulthood (Table 3). The ratios were found to fluctuate with the age of the insect. The alcohol components, I and II, were found in greater concentrations than the aldehyde components, III and IV. The ratio for component I was always greater than component II. On day 13, the largest drop occurred in the ratio for the alcohol components, while the concentrations of both aldehydes were equivalent. The pheromone ratio on that day was 2.41:2.29:0.95:1 for components I-IV, respectively. Hardee et al. (1974) found the most attractive ratio as 2.37:1.74:1.0:1.0 for components I-IV, respectively. They also established that the ratio of the components in the pheromone was more important for attracting boll weevils than the dose. Their most attractive ratio was very similar to the ratio we found on day 13. Tumlinson et al. (1969) determined that the boll weevil pheromone ratio was 12.6:9.5:1:1 by steam distillation of the frass. Their results, when compared to ours, suggest they sustained a loss in the aldehyde components during the prepurification stage with the steam distillation method. The pheromone ratios determined in our studies with the effluvial method are believed to be the true atmospheric ratios of the pheromone components produced by actively calling cultured boll weevils.

CONCLUSION

The effluvial method described in this report was found to be an efficient technique to capture boll weevil pheromone for quantification with capillary column gas chromatography. The two major advantages to the method are: (1) pheromone and other comingling odors are captured during active insect production and at biologically active concentrations, and (2) these potentially biologically active odors (pheromone and plant volatiles) are captured free of

TABLE 2. CONCENTRATION OF BOLL WEEVIL PHEROMONE COMPONENTS COLLECTED DURING DAY 6, 8, 10, 11, 12, 13, AND 14 OF ADULTHOOD

Day of adulthood	Conc. of pheromone components ($\mu\text{g}/\text{weevil}$) ^{ab}				Total pheromone ($\mu\text{g}/\text{weevil}$)
	I	II	III	IV	
6	0.10c	0.08c	0.04d	0.04d	0.26d
8	0.39b	0.35b	0.09cd	0.11c	0.94c
10	0.65ab	0.60ab	0.14bc	0.17bc	1.56bc
11	1.07a	1.03a	0.27abc	0.33abc	2.70abc
12	1.24a	1.19a	0.42ab	0.42ab	3.27ab
13	1.52a	1.44a	0.60a	0.63a	4.19a
14	0.90ab	0.87ab	0.34abc	0.30abc	2.41abc

^aI = (+)-*cis*-2-isopropenyl-1-methylcyclobutane ethanol; II = *cis*-3,3-dimethyl- $\Delta^{1,6}$ -cyclohexane ethano; III = *cis*-3,3-dimethyl- $\Delta^{1,6}$ -cyclohexane acetaldehyde; IV = *trans*-3,3-dimethyl- $\Delta^{1,6}$ -cyclohexane acetaldehyde; Means within columns followed by different letters are significantly different ($P < 0.05$, Duncan's multiple-range test).

TABLE 3. BOLL WEEVIL PHEROMONE COMPONENT RATIOS DURING DAYS 6, 8, 10, 11, 12, 13, AND 14 OF ADULTHOOD

Day of adulthood	Ratios of pheromone components ^a		
	I/IV	II/IV	III/IV
6	2.50bc	2.00b	1.00ab
8	3.55ab	3.18ab	0.82c
10	3.82a	3.53a	0.82c
11	3.24ab	3.12a	0.82c
12	2.95abc	2.83ab	1.00bc
13	2.41c	2.29b	0.95bc
14	3.00ab	2.90ab	1.17a

^aMeans within columns followed by different letters are significantly different ($P < 0.05$, Duncan's multiple-range test).

nonvolatile contaminants and thus are relatively pure and easily quantified. Furthermore, the effluvial method may be of value to entomological science for other reasons (Baker and Longhurst, 1981): (1) it provides a means to easily determine the ratios of pheromone components as they are found in the air surrounding actively calling insects; (2) it may be useful in the identification of geographic races of an insect pest; (3) in insect-pest-control programs utilizing sterile releases, pheromone production could be monitored with the method to evaluate the pheromone competitiveness between laboratory-reared insects and the natural field populations; (4) in studies of host-plant resistance, the effects of variety on pheromone production could be determined; (5) varieties might be developed that suppress pheromone production, thus being less attractive to insect pests; and (6) since plant volatiles from secondary metabolism are also involved in insect selection and colonization of host plants, the effluvial method of collection may be utilized by breeders in selection plants that have a less attractive aroma to insect pests.

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INSECT SEX PHEROMONES

Effect of Temperature on Evaporation Rates of Acetates from Rubber Septa¹

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Abstract—The half-lives ($t_{1/2}$) for evaporative loss of *n*-alkyl and *n*-alkenyl acetates from rubber septa were determined at temperatures varying from 15 to 35°C. The changes in $t_{1/2}$ with temperature gave high correlations with the equation, $\ln t_{1/2} = \Delta H/RT + y_0$ where ΔH is the heat of vaporization, R is the gas constant, T is the absolute temperature, and y_0 is a constant. Half-lives changed dramatically with temperature and the degree of change with temperature increased with increasing molecular weight. For mixtures, component ratios changed with temperature, but the degree was modest. At 20°C there was a 7.5-fold ratio of $t_{1/2}$ between members of the homologous *n*-alkyl or *n*-alkenyl acetates differing by two carbon atoms. The large change in $t_{1/2}$ with temperature and with number of carbon atoms is a consequence of the thermodynamic relationships and the temperature range of pheromone usage. Therefore, a similar degree of change in $t_{1/2}$ with temperature and number of carbon atoms will apply to other formulations of the same type (those in which the rate of evaporation is first order). The values of $t_{1/2}$ at 20°C mainly agreed very well with those reported previously at room temperature. However, our previously reported values for pentadecyl and hexadecyl acetate were revised. Half-lives were shown to depend on the vapor pressure of a compound in the formulation substrate, but not on the vapor pressure of the pure compound.

Key Words—Insect sex pheromones, controlled release, formulations, formulation substrates, acetates, *n*-alkyl acetates, *n*-alkenyl acetates, heat of vaporization, enthalpy of vaporization.

¹ Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

INTRODUCTION

The natural rubber septum (Roelofs et al., 1972) is the most commonly used controlled release substrate for insect sex pheromones in monitoring traps. In previous work from this laboratory, it was established that pheromones evaporate from rubber septa by a first-order process (Maitlen et al., 1976). Also, correlations of chemical structure of pheromones and analogs with half-lives from rubber septa were established, making possible calculations of evaporation rates of single compounds and vapor component ratios for mixtures containing acetates and/or alcohols (Butler and McDonough, 1979, 1981; McDonough and Butler, 1983). Olsson et al. (1983) reported a correlation of half-lives from rubber septa with vapor pressures as determined by a novel gas chromatographic method. Subsequently, Heath and Tumlinson (1986) and Heath et al. (1986) showed that half-lives from rubber septa correlate with vapor pressures and with gas chromatographic retention times from a liquid crystal liquid phase and that vapor component ratios from rubber septa of mixtures containing aldehydes, acetates, and alcohols could be predicted from their correlation. In other studies, evaporation rates or loss rates of sex pheromones from rubber septa of the pink bollworm (Flint et al., 1978), oriental fruit moth (Baker et al., 1980), the pea moth (Greenway et al., 1981), the red scale (Leonhardt and Moreno, 1982), and the olive fly (Jones et al., 1983) have been reported.

An important aspect not previously addressed is the influence of temperature on evaporation rates and on component ratios from rubber septa. Limited studies of the effect of temperature on evaporation rates for other controlled-release substrates have been reported. Bierl et al. (1976) reported the effect of temperature on the evaporation rates of disparlure from plastic laminate dispensers. Sower and Daterman (1977) studied the effect of temperature on the release rate of (*Z*)-6-heneicosen-11-one from plastic films, and Rothshild (1979) compared the release rates of (*Z*)-8-dodecen-1-ol acetate from several dispensers at three temperatures. More recently Leonhardt et al. (1988) reported the effect of temperature on the release rate of grandlure from different formulation substrates.

Our purpose was to develop a systematic study of the effect of temperature on evaporation rates and component ratios of homologous series of unsaturated and saturated acetates from red, natural rubber septa. Here we report our study showing that evaporation rates change dramatically with temperature while the change in component ratios with temperature is moderate.

METHODS AND MATERIALS

Method for Determining Half-Life. A pheromone or other test compound, impregnated in a rubber septum, was allowed to evaporate in a stream of nitro-

gen, and the evaporated pheromone was collected on an adsorbent (McDonough and Butler, 1983). The septum and adsorbent were individually extracted, and the amount of pheromone from each was determined. The half-life, $t_{1/2}$, was calculated from the equation:

$$t_{1/2} = \frac{P \ln 2}{R} \quad (1)$$

where R is the rate of evaporation (i.e., the amount of pheromone collected per unit of time on the adsorbent) and P is the amount of pheromone remaining in the septum.

Apparatus. A schematic drawing of the apparatus is shown in Figure 1. Nitrogen, purified by passing through a column of reverse phase (octadecyl-silica gel), passed through a flowmeter (Gilmont Instruments/Van Rogers & Waters, Seattle, Washington) and into the apparatus consisting of two 10-ml syringes sealed at the large ends with a rubber gasket and held together by a clamp (not shown). A Sep Pak liquid chromatographic cartridge (Waters Associates, Milford, Maine) containing RP-18 (Cat. No. 5190) was connected to the small end of the second syringe for collection of evaporated pheromone.

Procedure for Determining Half-Life. A red, natural rubber septum (West Co., Phoenixville, Pennsylvania) impregnated with the test compound (10–15 mg) was allowed to age for three or more days to allow any possible surface-deposited material to evaporate, and then placed in the syringe closest to the Sep Pak in the described apparatus (Figure 1). The nitrogen flow rate over the septum was 20 cm/sec, and the collection period was 1–12 hr, depending on the volatility of the test compound. After a run, the septum was removed and extracted for 4 hr with 50 ml dichloromethane, reextracted overnight with 50 ml more of dichloromethane, and the solutions were combined. Then 30 ml of heptane was added and the solution was concentrated to about 25 ml by rotary evaporation and made up to the desired volume with heptane. The compound in the Sep Pak was eluted by adding 20 ml of dichloromethane–methanol (9:1) in portions to the syringe to which the Sep Pak was attached and forcing the solvent through with the syringe piston. To the dichloromethane–methanol solution, 6 ml of heptane was added, and the resulting solution was concentrated to 5 ml with a rotary evaporator.

The solutions were analyzed by gas chromatography. The value of each determination of the half-life was the average of two values, one made on non-polar (methyl silicone, DB-1) and the other on polar (polyethylene glycol, DB-



FIG. 1. Apparatus for determination of half-life by collection of emitted vapor.

Wax) 15 m, megabore columns (J & W Scientific, Inc., Rancho Cordova, California).

The values of the half-lives of each compound were determined three times at each of three temperatures. For the 10- to 14-carbon acetates, the temperatures were 15, 22.5, and 35°C. For the 15- and 16-carbon acetates, the temperatures were 20, 27.5, and 35°C. The temperature was controlled by placing the entire apparatus in an insect environmental control chamber (model M-31, Environmental Growth Chambers, Chagrin Falls, Ohio), which controlled the temperature within $\pm 0.2^\circ\text{C}$. Temperature was monitored with a thermometer meeting National Bureau of Standards specifications (accuracy = $\pm 0.3^\circ\text{C}$).

Recovery Experiments. The ability of the Sep Pak cartridge to adsorb and retain pheromone vapors was determined by adding 20 or 50 μl of a dichloromethane solution of 1 $\mu\text{g}/\mu\text{l}$ of a test compound to the Sep Pak cartridge, rinsing it into the cartridge with another 50 μl dichloromethane, connecting the cartridge to the apparatus in the temperature control chamber, and passing nitrogen through it at the usual flow rate for various periods of time. The test compounds, the conditions, and the percent recoveries are summarized in Table 1.

Statistical Calculations. For the modified Clausius-Clapeyron equation,

$$\ln t_{1/2} = \frac{\Delta H}{RT} + y_0 \quad (2)$$

ΔH is the heat of vaporization (calories/mole), R is the gas constant (1.98 calories/degree), T is the absolute temperature ($^\circ\text{K}$), and y_0 is a constant. The ΔH values and 95% confidence limit of ΔH were calculated from the regression analysis of $\ln t_{1/2}$ versus $1/T$ from the expression:

$$\frac{\Delta H}{R} \pm \frac{t(n-2, 1-\frac{1}{2}\alpha)s}{[\sum(X_i - \bar{X})^2]^{1/2}}$$

where $t(n-2, 1-\frac{1}{2}\alpha)$ is the $(1-\frac{1}{2}\alpha)$ percentage point of a t distribution with $(n-2)$ degrees of freedom (the number of degrees of freedom on which the

TABLE 1. RECOVERY OF ACETATE PHEROMONES FROM C_{18} SEP-PAK CARTRIDGES EXPOSED TO NITROGEN FLOW FOR VARIOUS TIMES AND TEMPERATURES

Acetate	Temp. ($^\circ\text{C}$)	Time (hr)	Replicates (N)	Quantity (μg)	Recovery \pm SD (%)
(Z)-7-Dodecenyl	15	12	3	50	98.96 \pm 1.36
(Z)-7-Dodecenyl	32	4	3	50	99.71 \pm 0.29
Hexadecyl	20	24	3	20	97.64 \pm 2.00
Hexadecyl	40	12	3	20	97.63 \pm 0.85

estimate s^2 is based); $\Delta H/R$ is the slope; α is 0.05; and s is the residual variation (Draper and Smith, 1966).

RESULTS

Because of differing volatilities, the half-lives of the n -alkyl acetates were not all determined at the same temperatures. In order to compare half-lives of the series at the same temperatures, the nine $t_{1/2}$ values for each compound were subjected to regression analysis according to the modified Clausius-Clapeyron equation (equation 2). The parameters ΔH (in kilocalories/mole), y_0 , their 95% confidence limits, and the correlation coefficients (r^2), are summarized in Table 2 (unbracketed values). These parameters were then used in equation 2 to determine $t_{1/2}$ values for each compound at temperatures of 15, 20, 25, 30, and 35°C. Values of $t_{1/2}$ at each of the chosen temperatures were then analyzed according to the equation:

$$\ln t_{1/2} = aN + b \quad (3)$$

where N is the number of carbon atoms in the alkyl group of the homologous series of the n -alkyl acetates. The correlation coefficients (r^2) for these analyses were >0.99 .

Equations 2 and 3 represent two different relationships for defining $t_{1/2}$ values. If one assumes the validity of these equations and that deviations from them are a result of experimental error, then it is possible to improve the $t_{1/2}$ values by reiteratively calculating them from the regression lines of equation 2, equation 3, equation 2, . . . , etc., until a set of values is arrived at that fall exactly on the regression lines for both equations (for these new parameters of equation 2 see the bracketed values in Table 2). The values of the equation 3 parameters are given in Table 3. Therefore, the bracketed parameters of Table 2 and the values of Table 3 define $t_{1/2}$ values that correlate perfectly with both equations 2 and 3. The bracketed parameters of Table 2 are within the 95% confidence limits of the values calculated directly from the original experimental data in every case except for 10:Ac where the values are still quite close.

The compounds Z7-12:Ac and Z9-14:Ac also constitute a homologous series. When the ratios of $t_{1/2}$ for these compounds (relative to the adjusted $t_{1/2}$ values of the saturated acetates, Table 2) were calculated at 15, 25, and 35°C, no discernible trend in ratios was evident. These ratios were averaged (0.709 ± 0.026). This average ratio was then used to calculate half-lives at 20°C from the values of the saturated analogs for the compounds Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac. The half-lives so obtained were substituted into equation 3 using the parameters a and b of the saturated analog, and the N values at 20°C were calculated. The N values are the equivalent chain length

TABLE 2. PARAMETERS FOR MODIFIED CLAUSIUS-CLAPEYRON EQUATION ($\ln t_{1/2} = \Delta H/RT + y_0$) AND 95% CONFIDENCE LIMITS^a

Compound	ΔH (kcal), experimental (95% confidence limit) [reiterative regression]	y_0 experimental (95% confidence limit) [reiterative regression]	r^2 experimental [reiterative regression]
10:Ac	14.946 (13.73 to 16.16) [16.19]	-24.1278 (-26.20 to -22.05) [-26.3007]	0.9918 [1.0000]
11:Ac	17.758 (16.46 to 19.06) [17.36]	-28.0252 (-30.25 to -25.80) [-27.3079]	0.9933 [1.0000]
12:Ac	19.754 (18.32 to 21.19) [18.53]	-30.3981 (-32.85 to -27.95) [-28.3150]	0.9935 [1.0000]
13:Ac	19.828 (18.65 to 21.00) [19.70]	-29.6185 (-31.63 to -27.61) [-29.3221]	0.9947 [1.0000]
14:Ac	20.959 (19.65 to 22.27) [20.87]	-30.4959 (-32.74 to -28.25) [-30.3292]	0.9941 [1.0000]
15:Ac	22.027 (21.29 to 22.77) [22.04]	-31.2324 (-32.49 to -29.97) [-31.3363]	0.9983 [1.0000]
16:Ac	22.611 (20.73 to 24.50) [23.21]	-31.3567 (-34.50 to -28.21) [-32.3434]	0.9896 [1.0000]
Z7-12:Ac	18.72 (16.68 to 20.76) [18.13]	-28.9361 (-32.42 to -25.45) [-27.9661]	0.9854 [1.0000]
Z9-14:Ac	19.71 (18.28 to 21.13) [20.47]	-28.6930 (-31.12 to -26.26) [-29.9837]	0.9883 [1.0000]
Z11-16:Ac	ND [22.80]	ND [-31.9903]	ND [1.0000]
Z7-10:Ac	ND [16.07]	ND [-26.1984]	ND [1.0000]
Z9-12:Ac	18.76 (16.43 to 21.08) [18.41]	-28.7965 (-32.78 to -24.82) [-28.2061]	0.9811 [1.0000]
Z11-14:Ac	17.68 (13.08 to 22.28) [20.75]	-25.0317 (-32.93 to -17.14) [-30.2236]	0.9514 [1.0000]
Z13-16:Ac	ND [23.08]	ND [-32.2352]	ND [1.0000]

^aUnbracketed values of the saturated acetates were calculated directly from the experimental data. Bracketed values were derived to be consistent with the regression lines for the equation $\ln t_{1/2} = aN + b$ and the Clausius-Clapeyron equation (see text). ND signifies not determined experimentally. Parameters for unsaturated acetates were calculated differently (see text). For Z7-10:Ac, Z11-16:Ac, and Z13-16:Ac, ΔH and y_0 were calculated from parameters for the other members of their homologous series (see text).

values relative to the saturated acetates, and were assumed to be constants over the temperature range under consideration (15-35°C). The N values were: Z7-12:Ac, 11.66; Z9-14:Ac, 13.66; and Z11-16:Ac, 15.66. The same procedure was used for the homologous series Z9-12:Ac and Z11-14:Ac and the relative retention ratio averaged 0.901 ± 0.097 and gave N values of: Z7-10:Ac, 9.90; Z9-12:Ac, 11.90; Z11-14:Ac, 13.90; Z13-16:Ac, 15.90. The

TABLE 3. PARAMETERS FOR $\ln t_{1/2} = aN + b$ AT GIVEN TEMPERATURES FOR *n*-ALKYL ACETATES^a

Temperature (°C)	<i>a</i>	<i>b</i>
15	1.04216	-8.34697
20	1.00721	-8.48142
25	0.973428	-8.61135
30	0.940763	-8.73700
35	0.909158	-8.85857

^a(*N* = number of carbon atoms in alkyl group). For *n*-alkenyl acetates the same parameters apply but *N* values are decreased relative to the saturated analogs. Thus, for Z7-12:Ac, Z9-14:Ac, Z11-16:Ac, *N* = 11.66, 13.66, 15.66, respectively. For Z7-10:Ac, Z9-12:Ac, Z11-14:Ac, Z13-16:Ac, *N* = 9.90, 11.90, 13.90, 15.90, respectively.

$t_{1/2}$ values for the unsaturated acetates from equation 3 at different temperatures were then used to obtain equation 2 parameters shown in Table 2. In every case, these parameters (bracketed values, Table 2) are within the 95% confidence limits of the values calculated directly from the original experimental data.

The bracketed parameters of Table 2 were used to calculate $t_{1/2}$ values of saturated and unsaturated acetates from 15 to 35°C at 5° intervals. These values are given in Table 4.

TABLE 4. HALF-LIVES OF *n*-ALKYL AND *n*-ALKENYL ACETATES AT VARIOUS TEMPERATURES, CALCULATED FROM BRACKETED PARAMETERS OF TABLE 2 OR PARAMETERS OF TABLE 3

Compound	$t_{1/2}$ (days)				
	15°C	20°C	25°C	30°C	35°C
10:Ac	7.96	4.91	3.07	1.96	1.26
11:Ac	22.6	13.4	8.14	5.01	3.13
12:Ac	64.0	36.8	21.5	12.8	7.78
13:Ac	181	101	57.0	32.9	19.3
14:Ac	515	276	151	84.2	47.9
15:Ac	1.46×10^3	755	399	216	119
16:Ac	4.14×10^3	2.07×10^3	1.06×10^3	553	295
Z7-12:Ac	44.9	26.1	15.5	9.33	5.71
Z9-14:Ac	361	196	108	61.2	35.2
Z11-16:Ac	2.90×10^3	1.47×10^3	760	402	217
Z7-10:Ac	7.18	4.44	2.79	1.78	1.15
Z9-12:Ac	57.7	33.3	19.6	11.7	7.11
Z11-14:Ac	464	249	137	76.7	43.8
Z13-16:Ac	3.73×10^3	1.87×10^3	960	504	270

DISCUSSION

Effect of Temperature on $t_{1/2}$ Values. The effect of temperature on $t_{1/2}$ is striking (Table 4). When the temperature is changed from 30 to 20°C, $t_{1/2}$ changes from 1.96 to 4.91 days (a factor of 2.5) for 10:Ac and from 553 to 2.07×10^3 days (a factor of 3.7) for 16:Ac. Therefore, if the temperature range in which a male insect responds to the pheromone is 10°C, then evaporation rates will vary in the range of these factors.

The rate of change of $t_{1/2}$ with temperature for a given compound is determined by its ΔH value. ΔH values for pheromones in a natural rubber matrix have not been reported previously, but ΔH values for some pheromones in the pure state have been reported (Hirooka and Suwanai, 1978; Olsson et al., 1983). For compounds in common with ours, the values of Olsson et al. and ours (in kcal/mol) are, respectively: 10:Ac, 17.27 and 16.19; Z7-12:Ac, 18.27 and 18.13; Z9-14:Ac, 21.37 and 20.47. For compounds in common with ours, the values of Hirooka and Suwanai and ours are: 12:Ac, 14.35 and 18.53; 14:Ac, 15.87 and 20.87; Z9-14:Ac, 17.23 and 20.47. Our values are from 0.14 to 1.08 kcal/mol smaller than those of Olsson et al. and from 3.24 to 5.00 kcal/mol larger than those of Hirooka and Suwanai. The difference in values of Olsson et al. and ours can reasonably be attributed to the difference in the condensed state in the two experiments (i.e., the pure liquid pheromone versus a solution of the pheromone in rubber). We cannot account for the large difference between our values and those of Hirooka and Suwanai. Olsson et al. have previously noted the large difference between their value and that of Hirooka and Suwanai for Z9-14:Ac (the only compound in common in their two studies) and suggested that the value of Hirooka and Suwanai was too small compared to other literature values for similar compounds.

Effect of Temperature on Component Ratios. Because ΔH determines the rate of change of $t_{1/2}$ with temperature for a given compound and ΔH increases with chain length (Table 2), the ratio in the vapor of components of different chain lengths will change as temperature changes. However, the effect of temperature on component ratio is fairly modest. To take an extreme example, if a septum contained 553 μg of 16:Ac and 1.96 μg of 10:Ac, at 30°C the ratio in the vapor of these components would be 50:50 and at 20°C the ratio would be 60:40 in favor of 10:Ac. In a less extreme and more likely situation, a septum containing 61.2 μg of Z9-14:Ac and 402 μg Z11-16:Ac would produce a ratio in the vapor of 50:50 at 30°C and 54:46 in favor of Z9-14:Ac at 20°C.

Criteria for Homologous Series that Obey Thermodynamic Relationships. A homologous series of compounds is one in which adjacent members differ only by a methylene group. For such a series, thermodynamic properties (such as ΔH) should differ between adjacent members by a constant amount determined by the methylene group, providing no additional intramolecular effects occur. For a short-chain alkyl series such as methyl, ethyl, and *n*-propyl, one

would expect additional intramolecular effects to occur because in each case there will be shielding of the acetate group by the methylene groups and methyl group (with a corresponding decrease in its effective polarity) for each member of the series (Kovats, 1965; McReynolds, 1966). As chain length increases, the shielding of the functional group will become constant and not increase as N increases. For n -alkyl acetates this occurs when $N > 3$ (McReynolds, 1966). For n -alkenyl acetates, the position of the double bond will affect half-life or gas chromatographic retention time. Its position relative to both the polar and nonpolar end of the molecule is significant. It is not known how close the double bond can be to the acetate without mutual influence occurring, but because of carbon bond angles, influence is negligible for double bonds $> \Delta 6$. For the series Z7-12:Ac, Z9-14:Ac, Z11-16:Ac, the double bond is sufficiently removed from the polar end of the molecule and is at a constant position relative to the nonpolar end of the molecule. Therefore, this homologous series should show a constant incremental thermodynamic change between adjacent members. This was the basis for calculating the parameters for Z11-16:Ac from data for Z7-12:Ac and Z9-14:Ac. These equivalent chain lengths are in reasonable agreement with those estimated by Heath and Tumlinson (1986) and by Heath et al. (1986) for Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac from their correlation with GC data (11.81, 13.66, and 15.62, respectively, versus our values of 11.66, 13.66, and 15.66). Z5-10:Ac was not included in this series. Both Z7-10:Ac and Z13-16:Ac were included in the series with Z9-12:Ac and Z11-14:Ac. Heath and Tumlinson (1986) reported the equivalent chain length by their GC method for just one compound of this series, Z9-12:Ac. Their value of 11.98 was similar to our determination of 11.90.

Comparison of $t_{1/2}$ Values with Earlier Values by Residue Method. In earlier work from this laboratory (Butler and McDonough, 1979) the $t_{1/2}$ values of the n -alkyl acetates and some unsaturated analogs were reported at room temperature. The values had been determined by the residue method and room temperature had been estimated at 22-23°C. For the majority of the compounds, the values corresponding to our present study at 20°C (Table 4) agree well with those earlier values. Considering the large effect of temperature on $t_{1/2}$ and that in the previous work temperature was not controlled, it is not surprising that the earlier values agree best with our present values at a slightly different temperature. However, there was a significant difference between the earlier values of the 15- and 16-carbon acetates and our present ones. Previously 16:Ac was reported to behave anomalously and to have a shorter $t_{1/2}$ than 15:Ac (earlier values at room temperature and present values at 20°C: 15:Ac, 1353 and 755; 16:Ac, 481 and 2.07×10^3). In our present study 16:Ac did not behave anomalously and agreed with the equation 3 relationship. The earlier reported anomaly may have occurred because of the difficulty in determining the small changes in residue level for compounds with long half-lives, and because of the lack of temperature control. However, there may be other factors

involved since Heath et al. (1986) also reported similar anomalous behavior for 16:Ac. They suggested that since 16:Ac is a solid at room temperature, a solid-liquid phase equilibrium could be established and would lead to lower estimated $t_{1/2}$ values.

Importance of Rubber Septa Data for Other Formulation Substrates. For the homologous series of saturated acetates at 20°C, one observes a 7.5-fold ratio in $t_{1/2}$ for members of the series differing by two carbon atoms, whereas in gas chromatography, typically one finds about a twofold ratio in retention time for members of the series differing by two carbon atoms. This difference is a result of the nature of the Clausius-Clapeyron equation (equation 2) and the difference in the temperature ranges at which gas chromatography is conducted and pheromones are used. Thus, if one uses the bracketed parameters of Table 2 to calculate the half-lives of 12:Ac and 14:Ac at 170°C, then the $t_{1/2}$ of 14:Ac is 2.0 times that of 12:Ac. This principle is further reflected by the changes in slopes for the equation $\ln t_{1/2} = aN + b$ at different temperatures as shown in Table 3 for the homologous series. As the temperatures increase, the slopes decrease.

A similar effect of change of magnitude of $t_{1/2}$ change with temperature occurs when comparing the change in $t_{1/2}$ at different temperature levels. For 16:Ac a 10° change in temperature from 170 to 160°C will increase $t_{1/2}$ by 1.8-fold whereas a 10° change from 30 to 20°C will increase $t_{1/2}$ by 3.7-fold.

Because the magnitudes of the variation of $t_{1/2}$ with temperature are a function of the laws of thermodynamics and the temperature range over which pheromones are used, similar magnitudes of the variation of $t_{1/2}$ with temperature and chemical structure will apply to other formulations using other formulation substrates of the same type. Formulation substrates of the same type are those that release pheromone by a first-order evaporation, such as PVC pellets (Daterman, 1974) or plastic film laminates (Leonhardt and Moreno, 1982). Open-ended microcapillaries release pheromone at a rate proportional to the square root of the vapor pressure (Brooks, 1980) and therefore are not of the same type. In this case the variation in evaporation rate with temperature will be determined by $\Delta H/2$.

Relationship of $t_{1/2}$ Values to Vapor Pressures. Vapor pressures of pure compounds have occasionally been used to account for evaporation rates from formulation substrates or GC elution rates. Although this may be a good approximation in a given circumstance, as a general principle it does not hold. The vapor pressure of a component in a formulation substrate or GC liquid phase determines the half-life or retention time, but it is not necessarily related to the vapor pressure of the pure compound.

In an ideal mixture, the vapor pressure of a component is directly proportional to the mole fraction of that component and the proportionality constant is the vapor pressure of that component in the pure form (Raoult's law). How-

ever, in the situations of concern here, the solute (pheromone component) is dissolved in a nonvolatile solvent (gas chromatographic liquid phase or formulation substrate such as a rubber septum) and an ideal mixture is not attained. Instead Henry's law would be expected to apply: the vapor pressure of a solute is proportional to its concentration, but the proportionality constant is empirically determined and is not the vapor pressure of the pure component.

Since in septa the rate of evaporation of a pheromone component is directly proportional to its concentration (first-order evaporation) and the evaporation rate is directly proportional to the vapor pressure, then vapor pressure is directly proportional to concentration. Therefore, a first-order evaporation from a nonideal mixture implies that Henry's law applies.

For Henry's law to apply, the solute molecules must be completely surrounded by solvent molecules (concentration less than about 10%). Then, as concentration changes, the molar free energy (ΔG) of the binding forces between the solvent molecules and the solute molecules does not change. The reason retention time or half-life may give approximate correlations with vapor pressure of pure compounds is that the free energy of the solute-solvent binding forces may be similar in the septum or GC liquid phase to the free energy of the molecule-molecule binding forces in the pure compound. Most pheromone components are of low polarity (long hydrocarbon chain terminated by one polar group) and therefore are somewhat comparable to rubber or nonpolar GC liquid phases. Polar GC liquid phases and polar formulation substrates are not expected to give even an approximate correlation with the vapor pressures of the pure compounds. For example, in a nonpolar GC liquid phase such as methyl silicone, alkenyl acetates elute before their saturated analogs (the alkenes have higher vapor pressures than the saturated analogs, the same order as observed for the pure compounds), while in a polar GC liquid phase such as polyethylene glycol, alkenyl acetates elute after their saturated analogs (alkenes have lower vapor pressure than their saturated analog, the opposite of the order observed for the pure compounds).

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HUMIDITY EFFECTS ON RESPONSE OF *Argas persicus* (OKEN) TO GUANINE, AN ASSEMBLY PHEROMONE OF TICKS

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Abstract—The assembly behavior of the tick *Argas persicus* Oken in response to guanine has been found to be humidity dependent. Nymphal and adult male *A. persicus* assemble on guanine-treated paper disks only at low relative humidities ($25 \pm 5\%$). Exposure of the ticks to high relative humidities ($85 \pm 5\%$) results in a gradual induction of a negative response to the pheromone.

Key Words—Tick, Argasidae, Ixodidae, *Argas persicus*, assembly pheromone, guanine.

INTRODUCTION

Three different categories of pheromones are involved in the chemical communication system of ticks: assembly pheromones, sex pheromones, and aggregation-attachment pheromones (Sonenshine et al., 1982; Sonenshine, 1985). Of these, assembly pheromones are the most widely distributed, being associated with both argasid and ixodid ticks (Leahy et al., 1973, 1975a,b; Leahy, 1979; Graf, 1975; Treverrow et al., 1977; Uspensky and Emel'Yonova, 1980; Petney and Bull, 1981). They are deposited on the tick's natural substratum off the host where, on contact, they arrest the ambulatory activity of wandering ticks, contributing to the formation of tight clusters of akinetic ticks. This behavior is believed to protect the species during stressful conditions of the

nonparasitic phases of its existence and to improve its chances of finding its host when these return to their previous habitats under favorable conditions (Leahy, 1979).

It has long been recognized that tick assembly pheromones are nonvolatile, insoluble in less polar solvents, and relatively stable in the environment (Leahy, 1975a). A prominent feature is their relative lack of specificity, eliciting responses from different life stages of the same species as well as those of other species (Leahy et al., 1975b; George, 1981; Petney and Bull, 1981). It has been suggested that this phenomenon implies the operation of a common ancestral pheromone system retained by stabilizing selection for a behavior that has continued to play an important role in ensuring the survival of the species (Petney and Bull, 1981). The recent identification in our laboratory of guanine as the common assembly pheromone of both argasid and ixodid species supports this view (Otieno et al., 1985).

The present work is based on a fortuitous observation of erratic responses of *Argas persicus* nymphs to guanine during a rainy period in Nairobi, Kenya. Earlier, Hefnawy (unpublished, cited in Sonenshine et al., 1982) working with the ixodid *Rhipicephalus appendiculatus*, had observed that the assembly responses of the adults of this tick to filter papers treated with water extracts of nymphal and adult excreta were confined to periods of dry weather. Unfortunately, details of this work were not published. The possible ecological implications of these observations prompted us to examine the effects of extremes of humidity on the responses of *A. persicus* to the assembly pheromone under controlled conditions, and herein we describe the results obtained.

METHODS AND MATERIALS

Nymphal (mixed stage) and adult male *Argas persicus* were obtained from a colony at the Hebrew University in Jerusalem, Israel. As in our previous study (Otieno et al., 1985), this species was used because its assembly response in bioassays is higher than those of other species (Leahy et al., 1975b; Leahy, 1979). Guanine used for the experiments was obtained from Sigma Chemical Company (Sigma white crystals grade).

Guanine solutions were prepared weekly by stirring 10-mg samples in 10 ml of double-distilled water for 16 hr at 22°C. The sample was centrifuged at the same temperature and the supernatant containing 0.7 mg guanine/100 ml (Otieno et al., 1985) was retained for bioassays.

A two-choice bioassay system was used to monitor the assembly responses of *A. persicus*. It comprised four 1.7-cm-diameter filter paper (Whatman No. 3) disks, two tests and two controls, arranged in diagonal pairs in a glass Petri dish (6 cm). These were placed on slide warmers (Fisher), incubated at 28°C,

and kept in the dark at $25 \pm 5\%$ or $80 \pm 5\%$ relative humidity. Humidities were controlled by ZnCl_2 and water, respectively, placed in a series of smaller dishes. Fifty microliters of guanine solution was applied using a syringe to each of the test disks, which were then allowed to dry in a stream of warm air before being placed in the Petri dish. The control disks were treated with double-distilled water only. Twelve *A. persicus* nymphs or adult males were placed in the center of the dish, and the assay system was left for 20–22 hr before counts of ticks assembled on treated and untreated disks were made. Each bioassay set comprised four replicates at the lower humidity and four at the higher humidity. At the end of each run, the ticks were returned to their storage containers, the Petri dishes thoroughly cleaned, and freshly treated paper disks were used for the next set of replicates. Assembly responses of ticks at the following physiological states were tested: (1) nymphal and adult male *A. persicus* from batches kept under ambient conditions for two to three weeks after moulting; (2) nymphal and adult male *A. persicus* from batches kept continuously either at $25 \pm 5\%$ or $80 \pm 5\%$ relative humidity at 28°C ; (3) nymphs from a freshly moulted batch assayed daily at the two humidities from the first day for 30 days.

RESULTS

Table 1 gives the data (pooled from 18 replicates) representing the assembly responses of nymphs and male adults, three weeks after moults, to a choice of guanine-treated and control paper disks. Significant differences in assembly

TABLE 1. RESPONSES OF *Argas persicus* NYMPHS AND MALE ADULTS KEPT FOR 3 WEEKS AFTER MOULT UNDER AMBIENT CONDITIONS TO CHOICE OF GUANINE-TREATED^a (T) AND CONTROL (C) PAPER DISKS AT $25 \pm 5\%$ AND $85 \pm 5\%$ RELATIVE HUMIDITY

	Total No. of ticks	Assembled on T	Assembled on C	χ^2
Nymphs				
Low humidity	216	198	18	150 ^b
High humidity	216	97	119	2.2, NS ^c
Adult males				
Low humidity	216	182	34	101 ^b
High humidity	216	95	121	3.1, NS ^c

^aDosage of $0.15 \mu\text{g}$ guanine/cm² of paper.

^b $P < 0.001$.

^cNS, not significant at 0.05 probability level.

preference between treated and untreated disks are observed at the lower humidity for both stages of the ticks. At the higher relative humidity, the differences in assembly responses were not significant, although data from successive batches of 3- to 4-week-old ticks consistently suggested a possible avoidance reaction to guanine-treated disks. A second set of assays was carried out using a batch of ticks that had been kept at the higher humidity for a longer period (six weeks) to see if the guanine avoidance response could be accentuated. Table 2, comprising assay data pooled from 22 replicates, summarizes the results obtained. Preferences for the untreated disks were significantly higher than for guanine-treated disks, clearly showing the development of a negative response to the pheromone.

During the course of the present study, preliminary observations with several batches of freshly moulted *A. persicus* nymphs or adults suggested a different pattern of response at this physiological stage of the tick. Accordingly, the responses of several batches of nymphs were monitored daily from the first day after the moult to see if a consistent set of behavior is demonstrated at the two humidity levels. Table 3 illustrates the pattern of results obtained. Recently moulted nymphs were indifferent to choices of guanine-treated and untreated paper disks at lower relative humidities. At the higher relative humidity, the ticks showed a brief positive response to guanine, followed by a period of indifference before the induction of a negative response. Observation of a batch of nymphs for eight weeks showed that no further change in behavior takes place after the development of a positive response to guanine at the lower humidity and a negative response at higher humidity level.

TABLE 2. RESPONSES OF *Argas persicus* NYMPHS AND MALE ADULTS, CONDITIONED AT LOW ($25 \pm 5\%$) OR HIGH ($85 \pm 5\%$) RELATIVE HUMIDITY FOR 6 WEEKS TO CHOICE OF GUANINE-TREATED^a (T) AND CONTROL (C) PAPER DISKS AT TWO HUMIDITIES

	Total No. of ticks	Assembled on T	Assembled on C	χ^2
Nymphs				
Low humidity	264	176	88	29.3 ^b
High humidity	264	63	201	72 ^b
Adult males				
Low humidity	264	226	38	134 ^b
High humidity	264	83	181	36.4 ^b

^aDosage of 0.15 μg guanine/cm² paper.

^b $P < 0.001$.

TABLE 3. ASSEMBLY RESPONSES OF *Argas persicus* NYMPHS FROM DAY 1 AFTER MOULT TO DAY 30 TO CHOICE OF GUANINE-TREATED^a (T) AND CONTROL (C) PAPER DISKS AT LOW ($25 \pm 5\%$) AND HIGH ($85 \pm 5\%$) RELATIVE HUMIDITY (ASSEMBLY DATA FOR EVERY 3 DAYS HAVE BEEN POOLED TOGETHER)

Days	Low humidity				High humidity			
	T	C	O/D ^b	χ^2	T	C	O/D ^b	χ^2
1-3	64	80	0	1.8, NS ^c	109	35		38 ^{c**}
4-6	78	65	1	1.2, NS	70	74		0.1, NS
7-9	82	60	2	3.4, NS	62	82		2.8, NS
10-12	86	58		5.4*	66	78		1.0, NS
13-15	61	83		3.4, NS	58	86		5.4*
16-18	81	63		2.3, NS	71	73		0.03, NS
19-21	85	58	1	5.1*	76	68		0.44, NS
22-24	109	35		38**	59	85		4.7*
25-27	112	28	4	50**	27	117		56**
28-30	112	22	10	60**	25	119		61**

^aDosage of 0.15 μg guanine/cm² paper.

^bTicks found either outside (O) the paper disks or dead (D).

^cNS, not significant at 0.05 probability level; * $P < 0.05$; ** $P < 0.001$.

DISCUSSION

Several adaptive roles ascribed to the tick assembly pheromone have been the subject of debate in the literature (Leahy, 1979; George, 1981; Petney and Bull, 1981; Sonenshine, 1985; Otieno et al., 1985). A consensus view is that the pheromone exerts a significant influence in arresting activity during times when the weather conditions outside the resting sites are unfavorable (low humidity, high temperature) and host animals are unlikely to be available, and this contributes to the formation of tight clusters to minimize water losses. In addition, it has been suggested that the pheromone functions to hold the ticks within the habitats to which the host animals return periodically, thus increasing the chances of encounter with their hosts (Leahy, 1979). These views are consistent with the finding that a sustained positive response to guanine is associated with continued exposure of the ticks to low relative humidities. Moreover, the selection of a semiochemical that is nonvolatile, present in relatively large amounts, and environmentally persistent is most appropriate for long-term performance needed for such purposes. However, a chemical with such characteristics would also create a major problem: how to release the tick from its positive influence when the weather conditions improve. Our findings show clearly the

development in *A. persicus* of a negative response to the semiochemical at the higher relative humidity. A similar trend was apparently observed by Hefnawy (unpublished, cited in Sonenshine et al., 1982) with *R. appendiculatus*, and it may turn out to be a general behavioral phenomenon among both the argasids and the ixodids, although the level of humidity at which the reversal in response takes place may vary from one species to another.

Recently moulted *Argus persicus* nymphs are largely indifferent to guanine (Table 3), apart from a brief positive response at the high relative humidity. Thus, sensitivity of the tick to the purine appears to be influenced by the availability of metabolic water, which is likely to be relatively high in recently moulted ticks, and is consistent with the pheromone's role as an assembly stimulus when conservation of water becomes an important factor. The brief positive response to guanine at the high humidity (Table 3, data for days 1-3) has been demonstrated by three different batches of recently moulted nymphs, but its significance, if any, is unclear.

To our knowledge, guanine represents the first example of a pheromone system that can play two opposite roles depending upon the environmental condition. The way this occurs is a matter of speculation. We suggest three alternative mechanisms: (1) at high humidity the pheromone may undergo a reversible change to a molecular species that elicits an avoidance response; (2) the information from the pheromone receptor, apparently present on the palpal tarsi (Leahy et al., 1975a), is interpreted against a background of messages from humidity receptors (Sonenshine, 1963) and the final response of the organism depends upon a summation of the two; (3) the pheromone receptor protein is moisture sensitive and readily hydrates at elevated humidities to give a conformation that interacts in a different mode with guanine, resulting in different information to the central nervous system. The first alternative appears to be least likely. Water extracts of guanine-treated filter disks left within the bioassay system at low and high humidities gave identical UV spectra and high-pressure liquid chromatograms. Moreover, solid-phase (KBr) IR spectra of samples of powdered guanine kept at low and high humidities were essentially identical, indicating that no significant shift in the equilibrium between N(7)H and N(9)H tautomers had occurred in the solid phase. In any case, the small free energy difference (~ 1 kcal/mol) between these tautomers (Pullman and Pullman, 1971) means that there would be a facile drift of the equilibrium position to the one most favored at the receptor site, irrespective of the initial position. In our view, one or the other of the remaining alternatives might be the mechanism by which guanine plays a dual semiochemical role. Carefully designed behavioral studies in conjunction with sensory physiological work would help shed some light on the question.

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FOLLOWING OF CONSPECIFIC AND AVOIDANCE OF
PREDATOR CHEMICAL CUES BY PINE SNAKES
(*Pituophis melanoleucus*)

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Abstract—The ability of hatchling pine snakes (*Pituophis melanoleucus*) to follow or avoid the chemical trails of conspecifics and a king snake (*Lampropeltis getulus*) on paper substrates was investigated in Y-maze experiments. Hatchlings entered the arm with the adult conspecific trail and avoided the arm containing the king snake trail at a frequency much greater than that due to chance. The data support the hypotheses that pine snakes follow the chemical trails of adult conspecifics and avoid the chemical trails of a predator.

Key Words—Chemical cues, predator avoidance, pine snake, *Pituophis melanoleucus*, odor trails.

INTRODUCTION

Trailing of conspecifics or prey by chemical means has been demonstrated in controlled laboratory experiments for snakes (Brown and Maclean, 1983; Chiszar et al., 1986; Ford, 1982; Ford and O'Bleness, 1986; Ford and Schofield, 1984; Gehlbach et al., 1971; Heller and Halpern, 1981) and for lizards (Cooper and Vitt, 1986a). Further, reptiles often respond to odors or those on applicators by increased tongue flicks (Cooper and Vitt, 1984, 1986b; Cooper et al., 1986). In some cases pheromonal communication is suspected because adult male skinks follow the trails of adult conspecific females, but not those of other males (e.g., *Eumeces laticeps*; Cooper and Vitt, 1986c). For reviews of the role of chemoreception in reptiles, see Burghardt (1980), Simon (1983), and Von Achen and Rakestraw (1984).

Earlier studies have shown the use of chemical abilities in several important contexts, including recognition of prey (Burghardt, 1973; Chiszar et al., 1986), detection of conspecifics (Cooper and Vitt, 1984), discrimination of male from female conspecifics (Cooper and Vitt, 1984), discrimination of conspecific and closely related syntopic congeners (Cooper and Vitt, 1986c), trailing conspecifics to find hibernacula (Brown and Maclean, 1983) or mates (Ford and O'Bleness, 1986), and differentiation of ophiophagous from nonophiophagous snakes (Weldon and Burghardt, 1979; Weldon, 1982). However, Weldon's (1982) studies dealt with changes in tongue-flick frequency. There has been no clear demonstration in snakes of discrimination of ophiophagous from nonophiophagous snakes. Snakes can distinguish conspecific from heterospecific odor trails (Ford and O'Bleness, 1986). Because some snakes are predators on other snakes, it would be advantageous for prey species to be able to detect the chemical trails of potential predators and avoid them. This would be particularly true for hatchlings that are more vulnerable to predators because of their small size. In this article I report experiments designed to examine choice discrimination by hatchling pine snakes (*Pituophis m. melanoleucus*) of the odor trail of conspecifics and of a heterospecific predator of pine snakes, the king snake (*Lampropeltis getulus*).

METHODS AND MATERIALS

Under appropriate state permits, pine snake eggs were collected from the Pine Barrens of southern New Jersey (Ocean, Cumberland, and Monmouth counties), and 200 hatchlings were hatched and maintained in the laboratory in 1986. Date of hatching was noted, so the exact age of each snake at testing was known. Snakes were maintained individually in plastic (30 × 15 × 9 cm) cages containing paper for shelter. Snakes were given the opportunity to drink water daily and to eat young laboratory mice once a week. Not all snakes had eaten, but there were no significant differences in behavior as a function of this variable. Snakes were sexed by hemipenis eversion, a reliable rapid procedure for young snakes (Schaefer, 1934; Fitch, 1960; Gregory, 1983; Gutzke et al., 1985).

To determine whether pine snakes could make directional responses to chemical trails, Y-maze experiments were conducted. The base arm of the Y maze was 1 m long and 15 cm wide, with 15-cm-high wooden sides. At the end of the base arm, two side arms (experimental arms, 45° angle from the base arm) were the same dimensions as the base arm. The floor of the maze was covered with paper that was changed after each trial. Plexiglas was placed over the maze to prevent the snakes from escaping from the center, choice point of

the maze. All ends of the maze were open during testing, allowing the snake to leave the apparatus at the end of the trial.

There were four experiments: (1) control ($N = 30$, no chemical trail on either side), (2) hatchling conspecific versus no trail ($N = 74$, trail on one side), (3) adult conspecific (snout-vent length = 120 cm) versus no trail ($N = 44$, trail on one side), (4) adult king snake (snout-vent length = 86 cm) versus no trail ($N = 117$, trail on one side). Trials were conducted from September 25 to October 15 on hatchlings that were 25–35 days old. Each snake was tested once in either the control or predator experiment and once in either the hatchling or adult conspecific experiment. The order of the experiments was selected randomly each day. Snakes were assigned randomly to each experiment. Sample size varied because snakes were used only in one type of conspecific test. I was primarily interested in their responses to hatchlings (that they would encounter in nature) and the predator; thus I used more snakes in these two tests. On some trials a snake would not enter the test (they had recently eaten or were otherwise overactive), accounting for further unequal sample sizes. The temperature was maintained at 27°C during all experiments, which were conducted from 1100 to 1400 hr.

The base arm contained no chemical trail, and there was no odor trail in one of the experimental arms. A chemical trail in the other experimental arm was produced by letting the stimulus (hatchlings, adult pine snake, or king snake) move freely over the paper and shavings for 10 min while both ends of the experimental arm were closed. The hatchling trail was established by allowing three hatchlings from a brood not otherwise included in the sample to move freely over the experimental arm. Only one king and three pine snake adults were used for the odor trail because that is all that were available. I do not believe that these adults possessed an idiosyncratic odor different from other conspecifics. The chemical trail extended the full length of the experimental arm: the trail-providing snakes were seen to move the entire length of the arm. When handling snakes, paper, or shavings, the experimenters wore disposable surgical gloves to avoid imparting human or snake odors. Following each trial, the paper and shavings were changed, and the location of the experimental arm was changed. Whenever a snake contacted the sides of the maze, all walls were subsequently washed with soap and water. After every 10 trials the entire apparatus and floor were also cleaned with soap and water.

Each snake was placed by hand into the base arm facing the opening with its head at the entrance and allowed to move freely up the arm to the intersection, where it usually moved into one of the experimental arms. The trial ended when the snake left the *Y* maze, usually through the experimental arms, although some went back out the base arm after arriving at the *Y* junction. The time each snake was in the base and experimental arm, time at the *Y* junction, and the

number of tongue flicks when the snake was in each location were recorded. An assistant released the snake into the maze. The behavior of hatchlings in conspecific tests did not differ as a function of their choice; however, behavior did vary in the predator test, and these data are presented. Chi square tests were used to determine the significance of the responses frequency in the four experiments, and Kruskal-Wallis chi square tests were used to determine differences in central tendencies of time and tongue flicks under several conditions. Data are presented as means \pm 1 SD in the text.

RESULTS

Most snakes moved rapidly to the Y junction, remained motionless for a few seconds ($\bar{X} = 18.4 \pm 6$ sec), explored the Y junction for 5–35 sec ($\bar{X} = 19.9 \pm 11.2$ sec), and moved rapidly down one of the arms.

When there was no chemical trail (control experiment), there was no significant difference in their choice (Table 1). Pine snake hatchlings did not follow the chemical trails of other conspecific hatchlings, but they did follow the trails of adult pine snakes (Table 1). In these conspecific trials, only one snake turned around and went back out the base arm, and none tried to go over the top of the maze.

Hatchling pine snakes avoided the king snake chemical trail and chose the arm with no chemical trail (Table 1). In this experiment 100 of the snakes selected an arm, and 17 either turned around and went out the base arm ($N = 8$), tried to climb over the top displacing the Plexiglas ($N = 4$), or moved back

TABLE 1. RESPONSES OF *Pituophis melanoleucus* TO CHEMICAL TRAILS IN Y MAZE^a

Experiment	Arm contains	Number of times chosen	χ^2 (P)
Control	No trail (right)	13	0.53 (NS)
	No trail (left)	17	
Hatchling conspecific vs. no trail	Hatchling	44	2.64 (NS)
	No trail	30	
Adult conspecifics vs. no trail	Adult	38	23.3 (0.001)
	No trail	6	
Predator vs. no trail	King snake	11	60.8 (0.001)
	No trail	89	

^aIndividual snakes tested in only one of the conspecific tests, and either the predator or the control test. NS = not significant.

TABLE 2. BEHAVIOR OF NAIVE PINE SNAKES GIVEN A CHOICE OF PREDATOR TRAIL OR NO TRAIL^a

	Choice		$\chi^2(P)$
	King snake trail	No chemical trail	
Number of snakes	15	89	
Base arm			
Time (sec)	4.8 ± 0.9	4.4 ± 0.3	0.36 (NS)
Tongue flicks	18.0 ± 0.7	17.4 ± 0.2	1.79 (NS)
Choice site ^b			
Motionless			
Time (sec)	24.0 ± 11.7	13.4 ± 2.6	3.47 (NS)
Flicks	13.2 ± 4.2	18.7 ± 1.1	4.65 (0.09)
Explore			
Time (sec)	29.3 ± 19.6	9.9 ± 3.3	11.19 (0.003)
Flicks	4.2 ± 2.2	2.8 ± 0.7	8.32 (0.01)
Experimental arm			
Time (sec)	24.5 ± 10.7	4.1 ± 0.6	11.1 (0.003)
Flicks	16.1 ± .6	16.4 ± 0.3	1.36 (NS)

^aGiven are means ± 1 SD. Tongue flicks are per 15 sec. NS = not significant.

^b

and forth between the two experimental arms ($N = 5$). It appeared that some hatchlings were responding to airborne odors by their obviously increased activity and attempts to get out of the *Y* maze.

As might be expected, there were no differences in the time each snake was in the base arm as a function of its final choice (Table 2). However, snakes emitted significantly more tongue flicks when exploring at the *Y* junction and when they went down the king snake chemical trail compared to the arm with no chemical trail. Similarly, they explored longer when they selected the king snake arm and moved significantly more slowly down the king snake arm compared to the blank (Table 2).

There were no significant differences as a function of age (15–25 days old) or sex of the hatchling, time of day, or location of the experimental arm ($P < 05$).

DISCUSSION

Hatchling pine snakes detect and follow the chemical trails of adult conspecifics and avoid the chemical trails of adult king snakes. In nature, pine snake hatchlings emerge from their underground nests in September and find

hibernacula in the next few weeks (Burger and Gochfeld, 1985; Burger et al., 1988). New Jersey Pine snakes hibernate in underground burrows that they dig themselves (Burger et al., 1988), although elsewhere gopher snakes (*Pituophis melanoleucus deserticola*) hibernate in mammal burrows with other species of snakes (Parker and Brown, 1973). It would be adaptive for pine snake hatchlings to locate and follow the trails of adult pine snakes to such sites. In the field I have observed hatchling pine snakes shift direction and follow the trail left by a released adult pine snake. Recognition of a conspecific chemical odor is particularly adaptive in New Jersey where pine snakes hibernate in small groups of up to 14 individuals (Burger et al., 1988), in contrast to some other species of snakes which hibernate in coiled groups of several to hundreds (Brown et al., 1974; Aleksuik, 1976). Thus, a keen ability to recognize the chemical trail of one or two adults would be useful in quickly finding a hibernaculum. In the New Jersey Pine Barrens, adult pine snakes move back toward hibernacula in September, before some hatchlings have emerged (Burger and Zappalorti, unpublished data).

Failing to find an adult chemical trail, it might be adaptive for a hatchling to follow the trail of another hatchling, although hatchlings did not significantly select the hatchling trails over no trails. It may be that the trail left by only three hatchlings is insufficient to distinguish and choose, or that the trail of hatchlings is disadvantageous to follow because it represents the wanderings of another "naive" hatchling that has yet to reach a hibernaculum. This issue cannot be decided on the basis of present results (Burghardt, 1983).

In these experiments, pine snakes avoided the king snake chemical trail. King snakes prey on other species of snakes (Fitch, 1963; Ditmars, 1935), including pine snakes in the New Jersey Pine Barrens (R.T. Zappalorti, personal communication). Thus, pine snakes were avoiding a potential predator by avoiding the arm with the king snake trail.

Snake species respond defensively to ophiophagous snakes by body bridging (Weldon and Burghardt, 1979), biting (Klauber, 1972), head hiding in coils (Carpenter and Gillingham, 1973), freezing, and rapid escaping behavior (Weldon, 1982; Marchisin, 1980). Carpenter and Gillingham (1975) reported that the colubrid snakes they tested were unresponsive to king snakes during staged encounters, but they used only body bridging as an indication of response. More recently, Weldon (1982) showed that naive garter snakes (*Thamnophis elegans*) emitted significantly more tongue flicks to swabs rubbed against the skin of king snakes than to those from generally nonophiophagous snakes or blank swabs. Similarly, *T. sirtalis* emitted more tongue flicks to swabs rubbed against black racers (*Coluber constrictor*), another ophiophagous species, than to that of nonophiophagous snakes (Weldon, 1982). These studies clearly demonstrate a differential chemical response. Alternatively, one might suggest that such studies demonstrate recognition of familiar compared to unfamiliar scents (Chiszar et al., 1978).

In these experiments, hatchling pine snakes required longer to explore in the choice situation when they traversed the king snake odor trail. More of their responses were ambivalent in the king snake test compared to the other experiments. Furthermore, pine snakes gave significantly more tongue flicks to the king snake arm while exploring the choices, corroborating the findings of Weldon (1982) for other species. However, since only a subset of snakes selected the king snake arm, their behavior may be aberrant. Similar increases in tongue flicks emitted to ophiophagous snakes have been reported in lizards (*Lacerta vivipara*; Thoen et al., 1986). In total, these findings suggest that some snakes and other squamates can distinguish the chemical deposits of ophiophagous and nonophiophagous snakes and follow the trail of conspecifics.

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ATTRACTION OF SCOLYTIDS AND ASSOCIATED BEETLES BY DIFFERENT ABSOLUTE AMOUNTS AND PROPORTIONS OF α -PINENE AND ETHANOL

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Abstract—The attraction of bark and ambrosia beetles as well as associated beetles to α -pinene and ethanol was studied in field experiments with flight barrier traps. α -Pinene and ethanol were released individually and as combinations in approximately 1:1 or 1:10 ratios and at four different release rates. Ethanol attracted *Tomicus piniperda* (L.), *Hylurgops palliatus* (Gyll.), *Trypodendron lineatum* (Oliv.), *Hylastes cunicularius* Er., *H. brunneus* Er., *H. opacus* Er., and *Anisandrus dispar* (F.) (Scolytidae); *Glischrochilus quadripunctatus* (L.) and *Epuraea* spp. (Nitidulidae); *Thanasimus formicarius* (L.) (Cleridae); and *Rhizophagus depressus* (F.) (Rhizophagidae). α -Pinene attracted all these species with the exception of *T. lineatum*, *H. cunicularius*, and *A. dispar*. Combinations of α -pinene and ethanol resulted in synergistically increased attraction of all species with the exception of *H. opacus* and *A. dispar*. *A. dispar*, the only hardwood-associated species in the study, was repelled by α -pinene. Both the release rates and the ratio at which the two substances were released influenced the response of the beetles. The differences in response between the beetle species seem to reflect dissimilarities in the release of the two substances among the various types of breeding material to which the species are adapted.

Key Words—Coleoptera, Scolytidae, *Tomicus piniperda*, *Trypodendron lineatum*, *Hylurgops palliatus*, *Thanasimus formicarius*, host attraction, host volatiles, ethanol, α -pinene, synergism.

INTRODUCTION

Several scolytid species are known to utilize host volatiles such as monoterpenes and the degradation product ethanol when searching for suitable host

substrate. The release rates of terpenes and ethanol and the proportions in which they are released can be expected to vary greatly among various types of breeding substrate. Hence, the response of various scolytids to combinations of terpenes and ethanol can be expected to differ depending on their breeding substrate preferences. Earlier studies have demonstrated the effects of increasing the release rate of ethanol in combination with a fixed release rate of host terpenes on the attraction of scolytids and associated beetles (Klimetzek et al., 1986; Schroeder, 1988). However, no studies have been reported on the response of scolytids to combinations of terpenes and ethanol in which the release rates of the host terpenes are varied. α -Pinene was chosen to represent the terpenes in the present study since it is one of the major monoterpenes in both Scots pine, *Pinus sylvestris* L., and Norway spruce, *Picea abies* (L.) Karst, the two dominant conifers in Scandinavia.

The aim of this study was to compare the attraction of various scolytid species and associated beetles to combinations of α -pinene and ethanol in proportions of approximately 1:1 and 1:10 when released at four different rates. We were especially interested in establishing whether synergistic attraction occurs for any of the tested release rate combinations.

METHODS AND MATERIALS

All experiments were conducted in a 40-year-old Scots pine stand in the province of Uppland in central Sweden in 1987. The attraction of beetles to the different compounds or combinations of compounds was estimated using baited flight barrier traps (Schroeder, 1988; type b) modified from the type described by Chapman and Kinghorn (1955). Under the barrier (a 40 × 40-cm transparent plastic sheet) a funnel ending into a water-filled jar was hung. The chemicals used were (–)- α -pinene (Fluka 97%, $[\alpha]_D^{20} -42 \pm 3^\circ$) and 95% ethanol (5% water). The supply of α -pinene in the laboratory was stored at +4°C. Chemical analyses of samples of this supply before and after the experimental period revealed no detectable amounts of oxidation products. The analysis was performed on a Finnigan 4021 GC-MS instrument. The α -pinene contained 91% (–)- α -pinene (82% ee), and this analysis was performed on a α -cyclodextrin GC column.

The substances were released at different rates from polyethylene vials with different-sized openings. When combinations of α -pinene and ethanol were tested, the two substances were released from separate vials. When exposed to air, α -pinene autoxidizes to a variety of compounds (Borden et al., 1986). To minimize the release of these oxidized substances during the field experiments, new vials supplied with fresh compounds were applied (between 10 AM and noon) on each day of the experiment.

Experiment 1. The experimental design consisted of 10 blocks (replicates)

with 14 treatments (traps) in each block. An overview of the experiment and the release rates is given in Table 1. The traps in each block were placed 10 m apart in a circle. Within each block (circle) all traps were oriented in the same compass direction. The minimum distance between blocks was 15 m. The treatments were randomly assigned to the trap positions within each block. Baits were placed out on each of five days between April 23 and July 22. The positions of the treatments in the circle of traps were not changed during the experiment. The captured beetles were collected after each day and pooled for each trap in the analysis.

Experiment 2. In this experiment a particularly low release rate of α -pinene was tested (Table 1). The treatments were (1) unbaited control, (2) α -pinene, (3) ethanol, and (4) α -pinene plus ethanol. The experimental design was similar to that in experiment 1, but each block only consisted of four traps (treatments), and there were 14 blocks. This experiment was baited only on April 23.

TABLE 1. OVERVIEW OF TREATMENTS IN EXPERIMENTS 1 AND 2^a

Estimated release rate (mg/hr)					
α -Pinene			Ethanol		
\bar{X}	Range		\bar{X}	Range	
0.009	0.008-0.011				
0.11	0.11-0.12		0.10	0.08-0.11	
0.95	0.93-0.97		1.30	1.30-1.30	
10.53	10.42-10.65		9.73	9.13-10.33	
			128.1	108.4-141.1	
Experiment 1 treatment	α -Pinene (mg/hr)	Ethanol (mg/hr)	Experiment 2 treatment	α -Pinene (mg/hr)	Ethanol (mg/hr)
1	0	0	1	0	0
2	0.1	0	2	0.01	0
3	0.9	0	3	0	0.1
4	10.5	0	4	0.01	0.1
5	0	0.1			
6	0	1.3			
7	0	9.7			
8	0	128.1			
9	0.1	0.1			
10	0.1	1.3			
11	0.9	1.3			
12	0.9	9.7			
13	10.5	9.7			
14	10.5	128.1			

^aThe release rates of α -pinene and ethanol were estimated in the laboratory by measuring the weight loss of the vials over 6 hr when placed in a wind tunnel at 0.5 m/sec and 15°C. Three replicates in each sample.

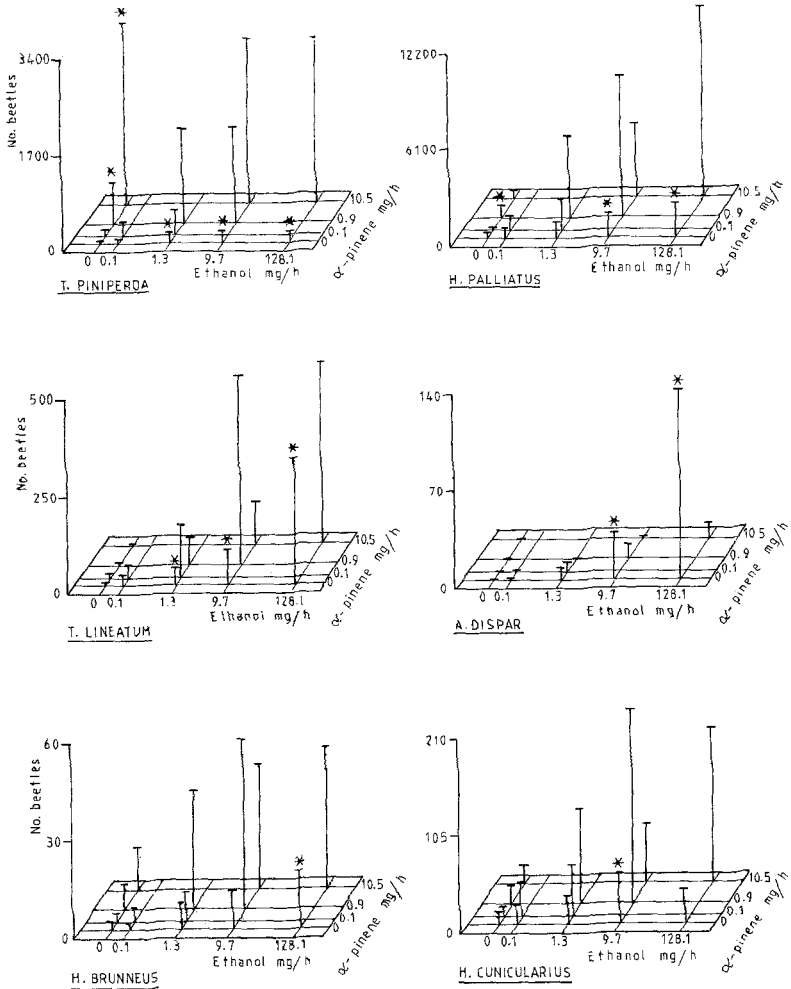


FIG. 1. Total number of beetles caught in flight barrier traps baited with α -pinene, ethanol, or both. Ten replicates of each treatment. Note the different scales of the catches and that the release rates have been transformed to $\log(x + 0.1)$ when arranged on the axes. An asterisk indicates a significant difference between the catch of treatment and that of the unbaited control at $P = 0.05$ level. Only those treatments in which α -pinene or ethanol were presented alone were tested for significant differences against the unbaited control.

When comparing the number of beetles caught in traps baited with α -pinene or ethanol at different release rates with the number caught in unbaited traps (Figure 1), data were analyzed with Friedman's test followed by a nonparametric test of Dunnett's type (Zar, 1984). Wilcoxon's signed rank test was used

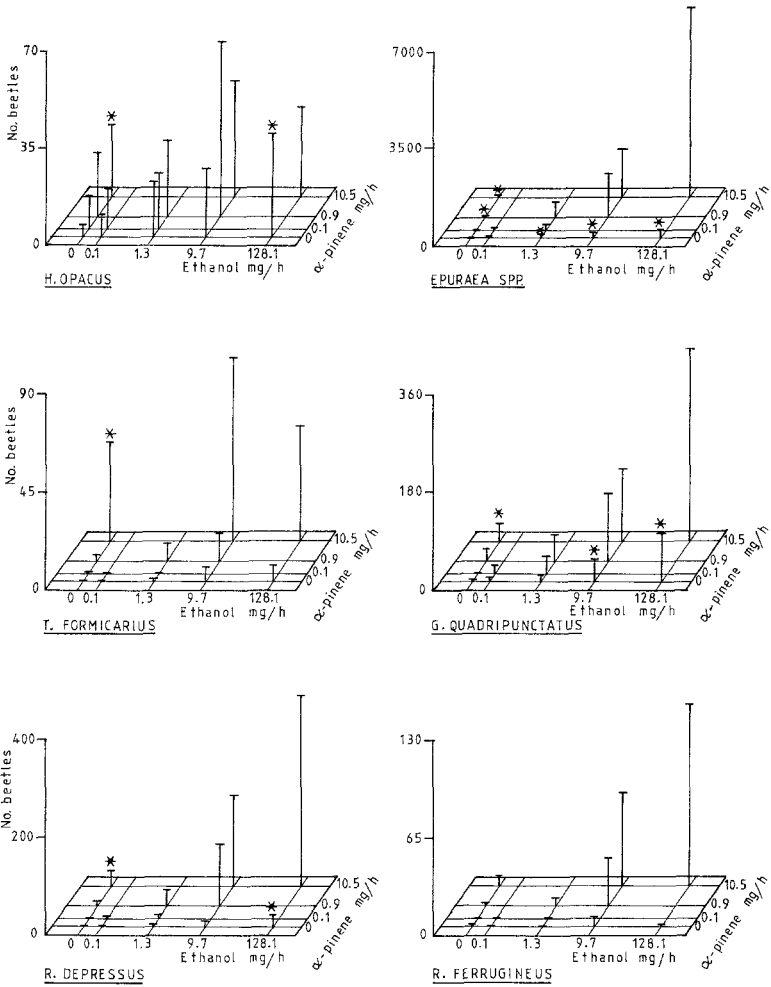


FIG. 1. Continued.

to test for synergism between ethanol and α -pinene (i.e., whether the combined treatment caught significantly more beetles than the sum of the two separate treatments).

RESULTS

The data from experiment 1 are presented in Figure 1, which gives an overview of the trap catches of the various species with regard to α -pinene, ethanol, and combinations of the two substances. Traps baited with α -pinene

alone caught significantly higher numbers than unbaited traps of the scolytids *Tomicus piniperda* (L.), *Hylurgops palliatus* (Gyll.), and *Hylastes opacus* Er.; the nitidulids *Epuraea* spp. and *Glischrochilus quadripunctatus* (L.); the clerid *Thanasimus formicarius* (L.); and the rhizophagid *Rhizophagus depressus* (F.). Catches of *Hylastes brunneus* Er. and *Hylastes cunicularius* Er. were also greater at α -pinene baits than in blank traps, but the differences were not significant. In contrast, the scolytids *Trypodendron lineatum* (Oliv.) and *Anisandrus dispar* (F.) were not caught in higher numbers in α -pinene-baited traps than in unbaited traps.

Traps baited with ethanol alone caught significantly higher numbers of all species than unbaited traps with the exception of *Rhizophagus ferrugineus* (Payk.) and *T. formicarius*. The catch of *T. formicarius* was higher at ethanol baits than in blank traps, but the difference was not significant.

Also in experiment 2, significantly more *T. piniperda* were caught in ethanol-baited traps ($\bar{X} = 7.8$) than in unbaited traps ($\bar{X} = 2.0$) (Wilcoxon signed rank test, $P < 0.05$). At the very low rate of α -pinene release used in this experiment, there was no significant difference in the catch of *T. piniperda* between α -pinene-baited traps ($\bar{X} = 3.3$) and control traps ($\bar{X} = 2.0$) and of *H. palliatus* between ethanol- ($\bar{X} = 2.2$) or α -pinene-baited traps ($\bar{X} = 0.4$) and control traps ($\bar{X} = 1.2$).

With the exception of *A. dispar* and *H. opacus*, the catches to the combinations of α -pinene and ethanol tested in experiment 1 were significantly higher for all species than the corresponding sums of the separate treatments, at one or more levels of release (Table 2). *A. dispar* was caught in the highest numbers in traps baited with ethanol alone, while traps baited with α -pinene alone caught no individuals. At the two higher α -pinene release rates, significantly fewer *A. dispar* were caught in the combined treatments than in the corresponding treatments baited with ethanol only (Wilcoxon signed rank test, $P < 0.01$).

α -Pinene and ethanol interacted synergistically in attracting *T. piniperda* at the two lower release rates of α -pinene. At the highest release rate of α -pinene, the combinations caught lower numbers (not significant) of *T. piniperda* than did α -pinene alone.

For *H. palliatus*, *T. lineatum*, *R. depressus*, *R. ferrugineus*, *Epuraea* spp., *H. cunicularius*, and *G. quadripunctatus*, synergism was strongest when the ethanol was released along with α -pinene at a rate approximately 10 times that of the latter. For *T. piniperda* and *H. brunneus*, the extent of synergism did not differ between combinations with an approximate 1:1 or 1:10 ratio between the release rates of α -pinene and ethanol.

In experiment 2 the catch at the combination of α -pinene and ethanol was significantly higher for *H. palliatus* ($\bar{X} = 4.4$), but not for *T. piniperda* ($\bar{X} = 14.7$), than the corresponding sum of the separate treatments (*H. palliatus*, $\bar{X} = 2.6$; *T. piniperda*, $\bar{X} = 11.1$) ($P < 0.05$, Wilcoxon signed rank test).

TALBE 2. SUMS OF BEETLES CAUGHT IN WINDOW TRAPS BAITED WITH α -PINENE ALONE, ETHANOL ALONE, AND NUMBER CAUGHT IN TRAPS BAITED WITH BOTH SUBSTANCES AT CORRESPONDING RELEASE RATES.

Species		α -pinene release rate (mg/hr)					
		0.1		0.9		10.5	
		Sum	Comb.	Sum	Comb.	Sum	Comb.
<i>T. piniperda</i>	A ^a	181	283 ^b	920	1714 ^b	3426	2919
	B	273	453 ^b	963	1685 ^b	3400	2935
<i>H. palliatus</i>	A	840	980	1812	5233 ^b	1996	4651 ^b
	B	1260	2036 ^b	2298	8945 ^b	2639	12063 ^b
<i>T. lineatum</i>	A	39	35	51	72 ^b	89	106 ^b
	B	56	140 ^b	95	481 ^b	230	462
<i>A. dispar</i>	A	1	1	8	0 ^b	32	1 ^b
	B	8	8	32	9 ^b	136	10 ^b
<i>H. brunneus</i>	A	5	5	16	36 ^b	25	38 ^b
	B	11	9	20	52	31	43
<i>H. cunicularius</i>	A	31	38	50	102	74	65
	B	41	57	73	208 ^b	56	168 ^b
<i>H. opacus</i>	A	20	15	44	28	52	43
	B	32	21	49	64	65	33 ^b
<i>T. formicarius</i>	A	1	0	4	8	51	85
	B	2	0	9	13	52	51
<i>G. quadripunctatus</i>	A	7	14	30	49 ^b	72	136 ^b
	B	11	32 ^b	59	127 ^b	118	354 ^b
<i>R. depressus</i>	A	3	4	12	34 ^b	40	188 ^b
	B	5	7	17	129 ^b	53	390 ^b
<i>R. ferrugineus</i>	A	0	0	1	4	12	63 ^b
	B	0	0	6	30	8	121 ^b
<i>Eपुरaea</i> spp.	A	43	81 ^b	122	493 ^b	230	1756 ^b
	B	81	220 ^b	221	1630 ^b	340	6894 ^b

^aA: release rates of α -pinene and ethanol are of the same magnitude; B: ethanol released at a rate approximately 10 times higher than that of α -pinene.

^bNumber of beetles caught by the combined treatment is significantly different from the sum of beetles caught by the separate treatments ($p < 0.05$; Wilcoxon signed rank test).

DISCUSSION

All scolytid species in the present study, with the exception of *A. dispar*, breed in conifers (Lekander et al., 1977) while the beetle species of other families occur together with various conifer scolytids (Nuorteva, 1956; Mills, 1983). *A. dispar*, which breeds in dead or dying trees of several hardwood species, was the only species in our study that was strongly repelled by the conifer monoterpene α -pinene. Similar results were earlier obtained for the scolytid

Trypodendron domesticum (L.), which also breeds in hardwood species (Nijholt and Schönherr, 1976). The strong attraction of *A. dispar* to ethanol alone has earlier been demonstrated by Magera et al. (1982) and Klimetzek et al. (1986). Moeck (1971) observed a strong attraction of *Anisandrus pyri* Peck. to ethanol alone.

In contrast to the other scolytids in this study, *T. piniperda* generally breeds in relatively fresh material, e.g., newly windbroken or windthrown trees and winter- or spring-cut logs (Trägårdh, 1921; Annila, 1975; Långström, 1984). Since such material has only just begun to deteriorate, the release of ethanol should be rather low, while high amounts of terpenes may be released from resin exuding from damaged parts. Accordingly, *T. piniperda* was the species most strongly attracted by α -pinene alone (see also Byers et al., 1985; Schroeder, 1988). Ethanol also attracted this species, but to a much lesser degree than α -pinene. Magera et al. (1982) caught higher numbers of *T. piniperda* in traps baited with ethanol (no release rates given) than in control traps, but the difference was not statistically significant. In a previous study (Schroeder, 1988), ethanol-baited traps did not catch higher numbers of *T. piniperda* than unbaited traps in two different experiments. But the number of *T. piniperda* caught in one of these experiments was low.

Vité et al. (1986) reported that up to 14 times more *T. piniperda* were caught in traps baited with a combination of racemic α -pinene plus terpinolene (1:1) and ethanol than in traps baited with the terpene mixture alone (no release rates given). The stronger synergistic effect in their study compared with the present one might be a result of differences between the ratios at which the host terpenes and ethanol were released in the two experiments and/or a result of differences between the monoterpenes baits used in the studies. However, there is no evidence in the literature suggesting that the differences in monoterpene baits between the present and the cited study should have influenced the results. In the study of Vité et al. (1986) a mixture of ethanol, racemic α -pinene, and terpinolene caught about the same number of *T. piniperda* as did a mixture containing the first two mentioned substances only (the terpenes were not tested alone in that experiment). Byers et al. (1985) caught about the same number of *T. piniperda* with (+)- α -pinene as they caught with (-)- α -pinene, and the combination of enantiomers did not have any synergistic effect on the attraction.

Although not significant, the decrease in attraction of *T. piniperda* that occurred when ethanol was added to the highest release rate of α -pinene in the present study is in agreement with the result of a similar experiment in a previous study (Schroeder, 1988). In that study the same α -pinene release rate (10 mg/hr) was combined with several ethanol release rates ranging from 7 times lower to 21 times higher than the release rate of the α -pinene. Each one of these combinations attracted fewer *T. piniperda* than did the α -pinene alone, and the difference was significant at the two highest ethanol release rates. Evidently,

maximal attraction of *T. piniperda* is attained by the α -pinene alone at this high release rate: attraction cannot be increased further by adding ethanol. Instead, high release rates of ethanol may decrease the attraction of *T. piniperda*, although the ratios between the substances are approximately the same as those that result in synergistic attraction at lower release rates.

Klimetzek et al. (1986) noted a fivefold decrease in catch of *T. piniperda* when increasing release rates of ethanol from 1 to 5 mg/hr (estimated in laboratory) in combination with a fixed release rate (not given) of the terpene mixture used by Vité et al. (1986).

H. palliatus, *T. lineatum*, *H. cunicularius*, *H. brunneus*, and *H. opacus* generally reproduce in dead or dying trees. The first three species prefer logs cut during the autumn of the previous year over newly cut logs (Trägårdh, 1921; Annila, 1975; Pulliainen, 1983). This type of older breeding material may release relatively high amounts of ethanol produced in deteriorating tree tissue (cf. Cade et al., 1970; Moeck, 1970), while monoterpenes are probably released in lower amounts compared with the amounts released from newly felled or broken trees (cf. Ikeda et al., 1980). None of the five scolytid species was attracted as strongly to α -pinene alone as was *T. piniperda*. The ambrosia beetle *T. lineatum* was not attracted at all by α -pinene and, for *H. cunicularius*, the difference in catch between α -pinene-baited traps and unbaited traps was too small to be interpreted as evidence of attraction to α -pinene. Ethanol strongly attracted *T. lineatum*, while attracting more weakly *H. palliatus* and the three *Hylastes* species. Ethanol alone has earlier been demonstrated to attract *H. palliatus* and *T. lineatum* (Moeck, 1970, 1971; Nijholt and Schönherr, 1976; Magema et al., 1982; Kohnle, 1985; Klimetzek et al., 1986; Schroeder, 1988).

Combinations of α -pinene and ethanol resulted in synergistic attraction in *H. palliatus*, *T. lineatum*, *H. cunicularius*, and *H. brunneus*. The lack of synergism in *H. opacus* is difficult to explain since this species inhabits a similar type of breeding material as the other two *Hylastes* species. Combinations with an approximate 1:10 ratio between the release rates of α -pinene and ethanol resulted in a stronger synergism than the corresponding 1:1 combination for *H. palliatus*, *T. lineatum*, and *H. cunicularius*. This illustrates that for these species the ratio at which the substances are released affects the beetle response.

Earlier studies with *T. lineatum* have demonstrated synergism in attraction between α -pinene and ethanol (Bauer and Vité, 1975; Nijholt and Schönherr, 1976), and data presented by Kohnle (1985) for *H. palliatus* suggest synergism between ethanol and spruce resin.

T. formicarius, *G. quadripunctatus*, *R. depressus*, *R. ferrugineus*, and *Epuraea* spp. are all species associated with bark- and wood-living beetles. With the exception of *T. formicarius*, adults of these species inhabit scolytid galleries. In this kind of substrate, deterioration processes should result in a gradual increase in the production of ethanol, released together with host ter-

penes. This could explain the strong synergistic effect of combinations of α -pinene and ethanol on attraction in these species. In contrast to the other species, *T. formicarius* adults prey on adults of several species of bark beetles, and among these is *T. piniperda* (Gauss, 1954; Nuorteva, 1956). The wide range of prey species, which in their turn are attracted to different kinds of breeding material, may explain the strong attraction of *T. formicarius* to α -pinene alone as well as to combinations of α -pinene and ethanol (see also Schroeder, 1988).

The present study demonstrates great differences between beetle species in their response to α -pinene, ethanol, and to combinations of the two. These differences probably reflect dissimilarities in the release of these volatiles among the various types of breeding material to which the species are adapted. Both the absolute release rates and the ratio at which the two substances were released influenced the response of the beetles to the combinations.

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CARDENOLIDE FINGERPRINT OF MONARCH
BUTTERFLIES REARED ON COMMON MILKWEED,
Asclepias syriaca L.

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Abstract—Monarch butterfly, *Danaus plexippus* (L.), larvae were collected during August 1983 from the common milkweed, *Asclepias syriaca* L., across its extensive North American range from North Dakota, east to Vermont, and south to Virginia. This confirms that the late summer distribution of breeding monarchs in eastern North America coincides with the range of this extremely abundant milkweed resource. Plant cardenolide concentrations, assayed by spectrophotometry in 158 samples from 27 collection sites, were biased towards plants with low cardenolide, and ranged from 4 to 229 $\mu\text{g}/0.1$ g dry weight, with a mean of 50 $\mu\text{g}/0.1$ g. Monarch larvae reared on these plants stored cardenolides logarithmically, and produced 158 adults with a normally distributed concentration range from 0 to 792 $\mu\text{g}/0.1$ g dry butterfly, with a mean of 234 $\mu\text{g}/0.1$ g. Thus butterflies increased the mean plant cardenolide concentration by 4.7. The eastern plants and their resultant butterflies had higher cardenolide concentrations than those from the west, and in some areas monarchs sequestered more cardenolide from equivalent plants. Plants growing in small patches had higher cardenolide concentrations than those in larger patches, but this did not influence butterfly concentration. However, younger plants and those at habitat edges had higher cardenolide concentrations than either older, shaded, or open habitat plants, and this did influence butterfly storage. There were no apparent topographical differences reflected in the cardenolides of plants and butterflies. Twenty-eight cardenolides were recognized by thin-layer chromatography, with 27 in plants and 21 in butterflies. Butterflies stored cardenolides within the more polar 46% of the plant R_f range, these being sequestered in higher relative concentrations than they occurred in the plants. By comparison with published TLC

cardenolide mobilities, spots 3, 4, 9, 16, 24 or 25, 26, and 27, may be the cardenolides syriocide, uzarin, syriobioside, syriogenin, uzarigenin, labri-formidin, and labriformin, respectively. Cochromatography with cardenolide standards indicated that desglucosyriocide did not occur in the plants but did occur in 70% of the butterflies, and aspecioside was in 99% of the plants and 100% of the butterflies. The polar aspecioside was the single most concentrated and diagnostic cardenolide in both plants and butterflies. Butterfly R_f values were dependent on those of the plant, and both showed remarkable uniformity over the range of areas sampled. Thus contrary to previous reports, *A. syriaca* has a biogeographically consistent cardenolide fingerprint pattern. The ecological implications of this for understanding the monarch's annual migration cycle are significant.

Key Words—*Asclepias syriaca*, biogeography, cardenolide fingerprint, chemical defense, *Danaus plexippus*, Lepidoptera, Danaidae, emetic potency, migration, milkweed, monarch butterfly, plant-herbivore interaction, predation, sequestration.

INTRODUCTION

The "common milkweed," *Asclepias syriaca* L., is the most abundant and widespread milkweed in northeastern North America and occurs in a wide diversity of open habitats (Woodson, 1954). Because the distribution of summer breeding monarch butterflies appears coincident with the geographical distribution of *A. syriaca*, this milkweed has been assumed to be the principal larval host plant of the monarch's eastern population (Urquhart, 1960, 1976; Urquhart and Urquhart, 1979). Furthermore, *A. syriaca* remains available as a food plant into the late summer when the last one or two generations of monarchs that migrate south are produced (Borkin, 1982; Malcolm et al., 1987). Consequently it is speculated that *A. syriaca* is the principal food plant of the autumn migrant monarchs that overwinter in Mexico (Fink and Brower, 1981; Brower et al., 1984a; Brower, 1985; Seiber et al., 1986).

Such speculation remains unsubstantiated, as the monarch's late summer breeding range has not been investigated but has been tautologically assumed to coincide with the distribution of *A. syriaca*. In this paper we present evidence that late summer breeding monarchs do in fact use this milkweed as a larval food resource across their range. We then describe the cardenolide storage patterns found in monarchs that have fed upon *A. syriaca* throughout this range.

Monarch butterflies store milkweed-derived cardenolides in ways that are characteristic of their larval host-plant species, making it possible to identify which plant species a wild, adult monarch had fed on as a larva (Roeske et al., 1976; Brower et al., 1982, 1984a,b; Cohen, 1985; Lynch and Martin, 1987;

Martin and Lynch, 1988; L.P. Brower, J.N. Seiber, C.J. Nelson, and S.P. Lynch, in preparation). Of the 108 North American species of milkweed in the genus *Asclepias* (Woodson, 1954), at least 28 are larval food plants of the monarch (Malcolm and Brower, 1986). Plant-butterfly cardenolide storage patterns, or "cardenolide fingerprints," for monarchs reared on eight of these milkweed species have been described, including six from California and two from Texas and Louisiana.

Since many of the North American *Asclepias* species have distinct geographical and temporal distributions, the fingerprinting technique allows a large-scale biogeographical analysis of the monarch's annual North American migration and breeding pattern through time and space. Fingerprinting wild-captured butterflies throughout their annual cycle can also be used to determine how monarchs exploit the various species of their milkweed hosts.

Recently, Seiber et al. (1986) found that the cardenolide aspecioside was present in 85% of overwintering monarchs in Mexico. Since this cardenolide has only been isolated from the two common northern milkweeds, *A. syriaca* and *A. speciosa* Torr., Seiber and his colleagues suggest that all overwintering monarchs with this cardenolide had fed as larvae on either or both of these milkweeds.

Although the presence of a single unique cardenolide is good evidence for what they termed, "the cardenolide connection" between northern, summer breeding monarchs and later, overwintering monarchs, knowledge of the cardenolide fingerprint and range of cardenolide concentrations across the breeding range of the butterfly is needed to provide a more reliable label of larval host plant origin.

No quantitative, detailed study of the cardenolide characteristics and fingerprint of *A. syriaca*-reared monarchs has previously been published. This is an obvious omission given the wide geographical range of this milkweed, the highly varied environments in which it occurs, and claims that it may have several races (Brower et al., 1982) and varying numbers of cardenolides (Roeske et al., 1976).

The purposes of the present study were to determine: (1) whether late summer breeding monarchs do use *A. syriaca* as a larval food resource across their range from North Dakota east to New England and south to Virginia (Figure 1); (2) the quantitative pattern of cardenolide sequestration and the cardenolide fingerprint of monarchs collected as larvae from *A. syriaca* across most of its range; (3) the influence of geographical area, topography, and habitat on cardenolide storage patterns; and (4) the reliability of the *A. syriaca* cardenolide fingerprint as a tool for biogeographical analyses of the monarch's annual migration cycle.

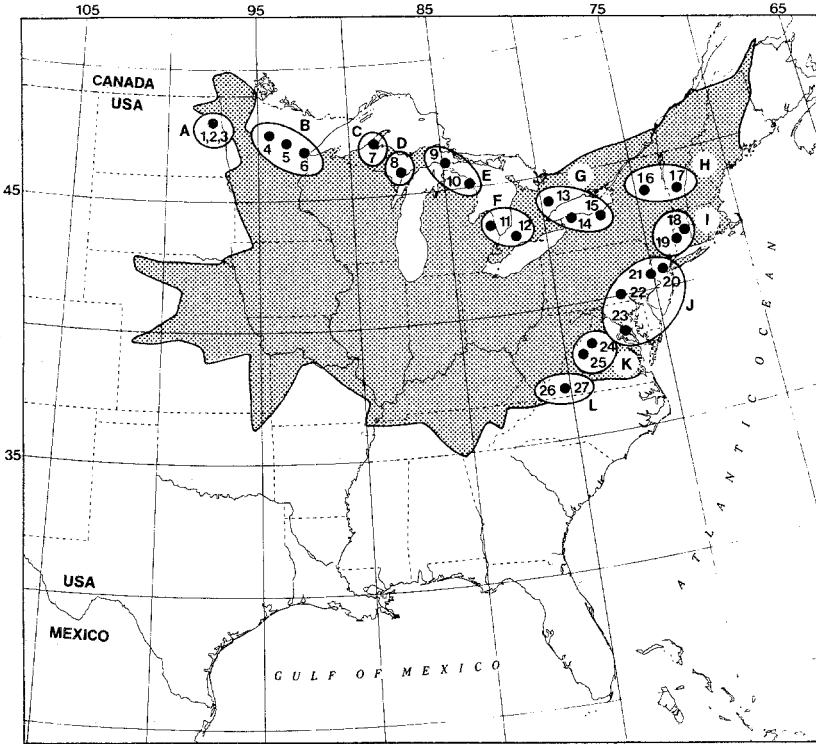


FIG. 1. Location of the 27 collection sites, during August–September 1983, grouped into the 12 areas, A–L, listed in Table 1, in relation to the distribution of *A. syriaca* (after Woodson, 1954).

METHODS AND MATERIALS

Distribution of Asclepias syriaca

We followed Woodson's (1954) distribution of *A. syriaca* to organize our collection of samples spanning the plant's range from its western limit in eastern North Dakota, east to New England and then south to the Appalachian Mountains in Virginia. Our 1983 field sampling showed that the distribution of *A. syriaca* agreed with that given by Woodson (Figure 1). Our subsequent field work in 1986 showed the presence of *A. syriaca* from southwest Missouri and east Kansas north through Nebraska and Iowa to Minnesota, thus confirming the western limits of Woodson's *A. syriaca* distribution. There is, however, no doubt that the species is considerably more common than Woodson indicated, since it occurs abundantly through Wisconsin and Michigan—areas that are largely devoid of location records on his map.

Location, Dates, and Methods of Sample Collections

Immature monarchs and leaf samples were collected from *A. syriaca* during the four-week period from August 7 to September 2, 1983, at the 27 sites in Table 1 and Figure 1, from North Dakota east to Vermont and south to Virginia. During sampling, we drove east and south with time so that the site numbers also indicate the order of collection. Sites consisting of *A. syriaca* patches were discovered en route, at approximately 150-km intervals, where we

TABLE 1. COLLECTION SITES AND SAMPLE SIZES OF TOTAL 158 PLANT-BUTTERFLY PAIRS, LISTED REGIONALLY (see A-L, Figure 1) AND BY 5 ECOLOGICAL GROUPS INCLUDING PLANT MATURITY, PATCH SIZE SAMPLED, HABITAT, POSITION, AND TOPOGRAPHY^a

Site No.	County/state	N	Area label	Maturity	Patch size	Habitat	Position	Topography
1	Grand Forks/ND	1	A	Mature	1	Open	Roadside	Flat
2	Grand Forks/ND	13	A	Mature	2	Shade	Pasture	Flat
3	Grand Forks/ND	1	A	Mature	2	Open	Roadside	Flat
4	Cass/MN	7	B	Mature	3	Edge	Roadside	Flat
5	Itasca/MN	3	B	Mature	2	Open	Roadside	Flat
6	St Louis/MN	1	B	Young	2	Open	Roadside	Lake
7	Houghton/MI	8	C	Mature	3	Shade	Pasture	Lake
8	Delta/MI	8	D	Mature	3	Open	Roadside	Lake
9	Mackinac/MI	5	E	Young	2	Edge	Roadside	Lake
10	Alpena/MI	15	E	Mature	3	Open	Roadside	Lake
11	Sanilac/MI	7	F	Mature	3	Open	Roadside	Lake
12	London/ON	5	F	Mature	1	Open	Roadside	Lake
13	Toronto/ON	1	G	Mature	1	Shade	Roadside	Lake
14	Niagara/NY	19	G	Young	1	Open	Pasture	Lake
15	Wayne/NY	1	G	Mature	3	Open	Roadside	Lake
16	Essex/NY	3	H	Mature	3	Shade	Roadside	Hilly
17	Windsor/VT	12	H	Mature	3	Open	Pasture	Hilly
18	Hartford/CT	2	I	Young	3	Edge	Roadside	Flat
19	Litchfield/CT	6	I	Mature	2	Edge	Roadside	Flat
20	Hunterdon/NJ	1	J	Mature	2	Open	Roadside	Flat
21	Hunterdon/NJ	3	J	Mature	1	Edge	Roadside	Flat
22	York/PA	5	J	Young	3	Open	Roadside	Flat
23	Charles/MD	4	J	Mature	1	Open	Roadside	Flat
24	Greene/VA	2	K	Mature	3	Shade	Roadside	Hilly
25	Nelson/VA	15	K	Young	1	Open	Pasture	Hilly
26	Franklin/VA	4	L	Mature	1	Edge	Roadside	Hilly
27	Patrick/VA	6	L	Mature	1	Shade	Roadside	Hilly

^aMilkweed patch sizes increase from 1 = <500 stems, to 2 = 500-1000, and 3 = >1000 stems per patch searched. Topographical proximity to one of the Great Lakes is indicated by "Lake."

counted stem numbers per patch and searched intensively for larvae to estimate larval density per stem. The pairwise collection procedure established in Brower et al. (1982) was followed, with part of the plant material on which a larva was feeding stored on Dry Ice for subsequent analysis and the remainder kept refrigerated for rearing the larva to maturity. No pupae were found. Of the 158 individuals analyzed, 92 (58%) were collected as fifth instars, plus 15 (9%) fourth instars, 16 (10%) third instars, 23 (15%) second instars, 10 (6%) first instars, and 2 (1%) eggs. Early developmental stages were collected since bagging of plants (Brower et al., 1982) was not feasible. Larvae were reared on their respective leaves in 10-cm-diameter plastic containers at ambient temperature. After a monarch pupated on the lid, a screen cylinder was inserted in the container to allow space for the emergent adult to expand its wings. Approximately 24 hr after emergence, the adults were placed in numbered glassine envelopes and stored frozen for subsequent analysis.

We could not gather equal numbers of larvae from each site since some sites were unproductive and larval mortality occurred after collection. To produce adequate sample sizes, we grouped the 27 collection sites into the 12 circled areas, labeled A–L in Figure 1 and Table 1. The basis of this grouping was a posteriori and involved proximity of sampling sites and a minimum sample size of eight plant–monarch pairs. In all but two cases less than 150 km separated the sites within each area, and more than 150 km separated the 12 areas. We also separately grouped the 27 sites by the five characteristics shown in Table 1 to explore cardenolide variation based on ecological as well as geographical differences.

Cardenolide Analyses

Spectroassay. The cardenolide content of 81 female and 77 male monarchs and the 158 samples of their respective host-plant leaves were determined by spectroassay as described in Brower et al. (1982), after Brower et al. (1972, 1975). Dried plant samples were ground to a powder and extracted with 95% ethanol in a 10-ml volumetric flask. Butterflies were similarly treated except that each dried individual was weighed, its right fore and hind wing lengths were measured, and it was then ground in ethanol with a Janke & Kunkel Ultra Turrax tissue mixer. The cardenolide concentration of each plant and butterfly extract was measured in micrograms (equivalent to the cardenolide standard, digitoxin) per 0.1 g dry weight of sample, and total cardenolide per butterfly was calculated by multiplying concentration by butterfly dry weight.

We made two changes in technique: (1) using a Perkin Elmer 559A spectrophotometer, digitoxin was found to absorb maximally at 622 nm and not 626 nm, as in Brower et al. (1972, 1975). Thus all cardenolide peak absorbances, when reacted with alkaline 2,2',4,4'-tetranitrodiphenyl (TNDP), were read at

622 nm. (2) Since the plant samples proved to have low cardenolide contents, 1 g dry weight was extracted in 10 ml instead of the 0.2 g of earlier studies.

Cleanup of Cardenolide Extracts. Some ketones, pregnane steroids, and plant pigments react with alkaline TNDP, resulting in overestimation of spectroassayed cardenolide content, especially in plant samples containing low to moderate amounts of cardenolide (Brown et al., 1979; Carolyn Nelson, personal communication). For example, the red tailing at the top of TNDP-sprayed TLC plates illustrated by Moore and Scudder (1985) is probably produced by the reaction of TNDP with pregnane glycosides. These compounds can interfere with TLC analysis by obscuring coincident cardenolides, and pregnane-3,20-dione steroids can add to colorimetric estimates of cardenolide content because they absorb light maximally at 555 nm, near the maximum absorbance wavelength of 622 nm for cardenolides (Carolyn Nelson, personal communication). Therefore, to make controlled comparisons between plant and butterfly extracts, both were spectroassayed, then cleaned by lead acetate pigment precipitation (Brower et al., 1982, 1984a), and spectroassayed again before TLC analysis. We modified the cleanup method for both plants and butterflies from that of Brower et al. (1984a) as follows: (1) The aqueous ethanol supernatant after pigment precipitation was partitioned with 2 ml hexane, the hexane fraction was back extracted with 2 ml 1:2 95% ethanol-water, and then the aqueous ethanol layers were pooled. The hexane extraction was used to remove noncardenolide contaminants since the discarded hexane layer extracts low-polarity lipids and pigments, but not cardenolides (Reichstein, 1967). (2) Instead of partitioning the aqueous ethanolic extract twice with 2:1 chloroform-95% ethanol, it was partitioned three times to ensure cardenolide extraction. The salt removal process with the dried extract was done once, and then plant and butterfly samples were resuspended in 95% ethanol and spectroassayed once again. The remaining cleaned extract was dried under N₂. Salts were removed once again, the extract dried under N₂, and then stored frozen in a 0.5-dram vial for TLC.

Before cleanup, the mean cardenolide concentration of the 158 plant samples was 20 $\mu\text{g}/0.1$ g dry weight (SD = 27, range = 0-187 $\mu\text{g}/0.1$ g). Following cleanup, the mean increased to 50 $\mu\text{g}/0.1$ g (SD = 39, range = 4-229 $\mu\text{g}/0.1$ g). Although the lead acetate cleanup procedure increased the mean plant cardenolide concentration, rather than decreasing it as in previous studies (*A. speciosa* decreased by 0.12 times; Brower et al., 1984a), the range of cardenolide concentrations in cleaned and unclean extracts changed very little. The change in mean concentrations is difficult to assess at such low cardenolide levels near the threshold for detection, particularly with such a strongly skewed distribution. We have less confidence in the accuracy of the unclean spectroassays because interfering pigments were concentrated by the necessity of extracting five times as much plant material in the same volume of solvent as earlier

studies. Thus we used the cleaned plant cardenolide estimates in our analysis, whereas earlier studies used data for unclean samples.

In contrast to the plant material, the cleanup process had little effect on the values of butterfly cardenolide concentrations. Prior to cleanup, the 158 butterflies had a mean cardenolide concentration of $234 \mu\text{g}/0.1 \text{ g}$ dry weight (SD = 148, range = 0–779 $\mu\text{g}/0.1 \text{ g}$) and after cleanup, the mean remained the same (SD = 150, range = 0–792 $\mu\text{g}/0.1 \text{ g}$). Since there was almost a one-to-one correspondence between clean butterfly concentration (CBC) and unclean butterfly concentration (UBC), according to the linear regression equation, $\text{CBC} = 1.02 (\text{UBC}) - 3.58$; $r^2 = 0.99$, only cleaned butterfly values are used for consistency with the plants.

Thin-Layer Chromatography (TLC). The cleaned plant and butterfly extracts were spotted, separated, and visualized on Merck Silicagel 60 F₂₅₄ plates. Each plate was developed four times at 20°C in the solvent system chloroform–methanol–formamide (90:6:1), as described in Brower et al. (1982). Extracts were spotted in chloroform so that 20 μl of solvent contained an estimated 100 μg of plant cardenolide and 75 μg of butterfly cardenolide, providing that sufficient cardenolide was available. If it was not, all of the sample cardenolide was added to the plate. Plant and butterfly paired samples were run side by side. To control for plate-to-plate variation, the paired samples were spotted to spread butterfly sex and collection sites across the 27 plates.

As in previous studies, digitoxin and digitoxigenin were spotted (10 μg of each) in three channels on each plate, along with two additional cardenolide standards known to occur in *A. syriaca*, desglucosyriaside (Cheung and Watson, 1980; Cheung et al., 1980) and aspecioside (Cheung et al., 1986; Seiber et al., 1986).

Visualized cardenolide spot mobilities, relative to digitoxin (R_d values), spot intensities (SI), and their probabilities of occurrence (PO) were measured and calculated as described in Brower et al. (1982) from projected 35-mm color slides of the TLC plates.

Statistical Analyses

Statistical analyses were performed with SAS (1985). All data were tested for normality before applying parametric statistics. Plant cardenolide concentrations were converted to a normal distribution by \log_{10} transformation. Analysis of variance was used where discrete variables only were investigated; however, where butterfly variation could also be influenced by variation among the plants, we applied analysis of covariance. Where appropriate, we used sequential retesting (Freund and Littell, 1985) and dropped insignificant interactions to assess the significance of remaining effects. For clarity and compactness, we omitted insignificant interactions from the tables.

RESULTS

Distribution of Monarch Larvae on A. syriaca

Monarch larvae were collected across the late summer range of *A. syriaca* from North Dakota (August 7, 1983), east to Vermont, and south to Virginia (September 2, 1983), at the 27 sites, grouped in 12 areas, (Figure 1, Table 1). A total of 245 patches of *A. syriaca* was searched, of which 70% had no monarch eggs or larvae. The numbers of immatures in the remaining 74 patches varied between 1 and 24 at the 27 sites.

Cardenolide Concentrations

Plant Variation. Following cleanup, the range of 158 plant cardenolide concentrations was 4–229 $\mu\text{g}/0.1$ g dry weight with a mean of 50 $\mu\text{g}/0.1$ g and SD of 39 (Figure 2). These concentrations ranged from 0.004% to 0.23% of plant dry weight, with a mean of 0.05%. Many plants contained extremely low quantities of cardenolide, and 63% contained less than the mean concentration, producing a positively skewed distribution (Figure 2, moment of skewness = 1.53; mean/median = 50/42; $D = 0.16$, $P < 0.01$). Since \log_{10} -transformed data were normally distributed, they were used for all subsequent analysis of plant cardenolide concentration.

We tested for differences among plant cardenolide concentrations, with respect to the 12 regional areas, the sex, and the larval stage of the monarch that was collected from them, by analysis of variance (Table 2). Area had a significant effect on plant cardenolide concentration. Duncan's multiple-range test split the plant samples into six overlapping groups (Table 3), of which the eastern *A. syriaca*, from Connecticut (I), New Jersey, Pennsylvania, and Maryland (J), the Thumb of Michigan, and Ontario (F) had significantly higher cardenolide concentrations than the western plants from North Dakota (A), Minnesota (B), and upper Michigan (C). Cardenolide concentrations of the plants from the other Michigan sites, New York, Vermont, and Virginia (areas D, E, G, H, K, and L) lay between these extremes.

The westernmost plant sample was also the most northern, as well as the earliest we collected. However, using Kendall's rank correlation coefficient (τ), we found the ranking of milkweed sites by cardenolide concentration fitted an east–west gradient ($\tau = 0.73$, $P = 0.0005$) better than either a north–south ($\tau = 0.57$, $P = 0.005$), or a temporal one ($\tau = 0.58$, $P = 0.005$), although the east–west effect does contain a significant north–south element ($\tau = 0.59$ for Kendall's partial rank correlation coefficient between plant cardenolide concentration and east–west position, with north–south position held constant).

There was no significant difference in plant cardenolide concentration according to the sex of the monarch larva feeding on them (Table 2). Larval

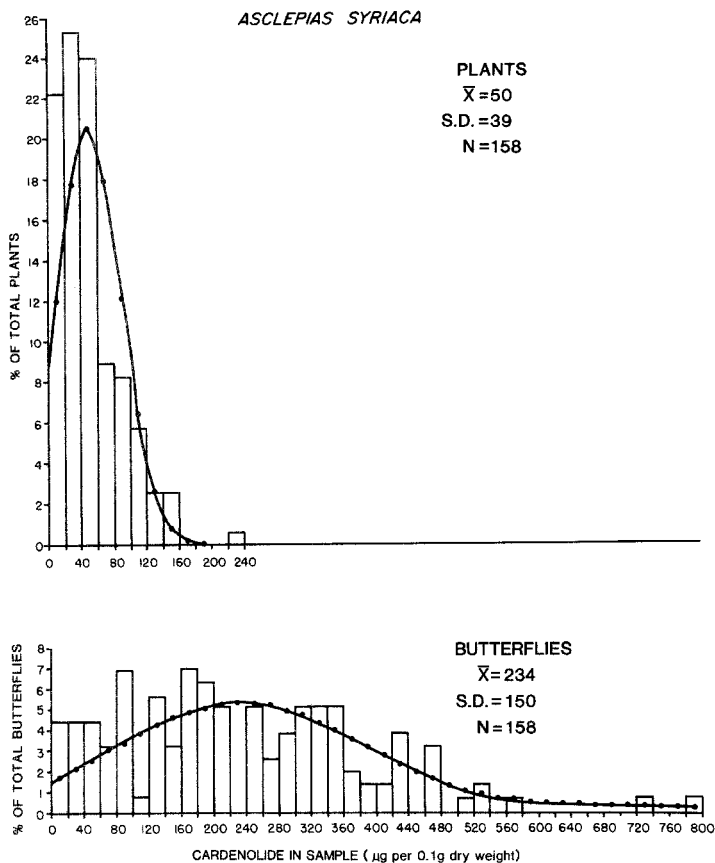


FIG. 2. Distributions of cardenolide concentrations ($\mu\text{g}/0.1$ g dry weight) of *A. syriaca* samples and their derived monarchs, collected from the locations illustrated in Figure 1. The curves are the calculated normal distributions.

TABLE 2. TWO-WAY ANALYSIS OF VARIANCE OF PLANT CARDENOLIDE CONCENTRATIONS BY AREA, SEX, AND INSTAR COLLECTED ($r^2 = 0.45$)^a

Source of variation	df	SS	MS	F	P
Model	37	8.09	0.22	2.67	0.0001
Error	120	9.81	0.08		
Corrected total	157	17.90			
Area	11	3.94		4.06	0.0001
Sex	1	0.08		0.88	0.3503
Instar	5	0.46		1.05	0.3933
Area \times instar	20	3.50		1.98	0.0126

^aArea by instar was the only significant interaction.

TABLE 3. PLANT CARDENOLIDE CONCENTRATIONS BY REGIONAL AREAS^a

Sample size	Plant cardenolide concentration ($\mu\text{g}/0.1 \text{ g}$)			Area	Duncan's grouping
	Mean	SD	Range		
8	75.7	1.7	43-158	I	1
12	54.7	1.8	27-158	F	1 2
13	54.6	2.3	18-229	J	1 2
17	49.6	1.8	18-110	K	1 2 3
15	45.9	1.6	16-100	H	1 2 3
21	42.1	1.8	14-116	G	2 3 4
10	35.6	3.0	8-137	L	2 3 4
20	33.6	2.3	7-137	E	2 3 4 5
8	28.4	1.9	10-56	D	3 4 5 6
15	24.4	2.1	5-100	A	4 5 6
8	19.4	2.8	4-75	C	5 6
11	16.2	2.1	5-48	B	6

^a See Figure 1 and Table 1. Areas with the same numbers are not significantly different by Duncan's multiple-range test at $P < 0.05$.

maturity at collection was also an insignificant main effect, but the interaction between area and larval stage was a significant determinant of plant cardenolide concentration. We think this interaction is significant because at some sites a particular instar predominated, causing instar and area effects to become compounded. This seems a more likely explanation than suggesting that larvae at different instars actively seek out plants of differing toxicity or are subject to differential mortality related to the host plant's cardenolide concentration, especially since larval instar at collection was not a significant main effect and had no significant effect on butterfly cardenolide concentration (see below).

Butterfly Variation. The 158 cleaned butterfly cardenolide concentrations were normally distributed over a range of 0-792 $\mu\text{g}/0.1 \text{ g}$ dry weight (Figure 2) with a mean of 234 $\mu\text{g}/0.1 \text{ g}$ (SD = 150, mean/median = 234/216, $D = 0.06$, $P = 0.13$). Three butterflies (1.9% of the sample) had no measurable cardenolide concentrations.

Variation in Butterflies as a Function of Plant Variation, with Geographical, Sex, and Instar Effects. The mean butterfly cardenolide concentration was 4.7 times that of the plants, and the butterfly cardenolide concentrations also had a considerably higher variance than the plants (Figure 2). For the whole data set pooled over sex, instar, and the collection sites, \log_{10} plant concentration predicted butterfly concentration with an r^2 of 0.61 ($P < 0.0001$; Table 4A, Figure 3). This logarithmic relationship means that butterflies store car-

TABLE 4. (A) REGRESSION OF BUTTERFLY CARDENOLIDE CONCENTRATIONS ($\mu\text{g}/0.1 \text{ g}$) AGAINST LOG_{10} PLANT CONCENTRATIONS, POOLING SEX AND COLLECTION SITES ($r^2 = 0.61$).
 (B) ANALYSIS OF COVARIANCE OF BUTTERFLY CARDENOLIDE CONCENTRATIONS ($\mu\text{g}/0.1 \text{ g}$) AGAINST LOG_{10} PLANT CONCENTRATIONS BY AREA, SEX, AND INSTAR, WITH SIGNIFICANT INTERACTION TERMS ($r^2 = 0.72$)

A. Source of variation	df	SS	MS	F	P
Plant concentration	1	2162759	2162759	243.1	0.0001
Error	156	1388010	8898		
Corrected total	157	3550769			
Estimated value of parameters		SE		t^a	P
Y intercept	-292.0	34.5		-8.5	0.0001
Slope	334.4	21.4		15.6	0.0001
B. Source of variation	df	SS	MS	F	P
Model	29	2566184	85539	11.0	0.0001
Error	128	984585	7753		
Corrected total	157	3550769			
Log plant concentration	1	1232995		159.0	0.0001
Area	11	185561		2.2	0.0196
Sex	1	47316		6.1	0.0148
Instar	5	15716		0.4	0.8743
Log plant conc. \times area	11	222732		2.6	0.0049

^aStudent's t for $H = O$, intercept or slope.

denolides more efficiently from plants of lower cardenolide concentrations but approach an upper limit with increasing plant cardenolide concentration.

A significantly improved fit to the data was obtained by applying a full analysis of covariance model that included sample area, sex, and instar at collection, in addition to the plant concentration effect ($r^2 = 0.72$, $P < 0.0001$, Table 4B). The larval stage at which an individual was collected had no significant effect on adult butterfly cardenolide concentration. Females had significantly higher cardenolide concentrations than males, with mean values of 262 $\mu\text{g}/0.1 \text{ g}$ and 204 $\mu\text{g}/0.1 \text{ g}$, respectively. The sexes had similar cardenolide concentration slopes, but the intercepts were significantly different, hence male cardenolide concentration = 329 (log_{10} plant concentration) - 300, and female cardenolide concentration = 329 (log_{10} plant concentration) - 269. This ability of the females to convert a particular plant concentration to a higher butterfly concentration has been found for monarchs reared on several other *Asclepias* species (Brower et al., 1972, 1975, 1982; Brower and Glazier, 1975; Brower

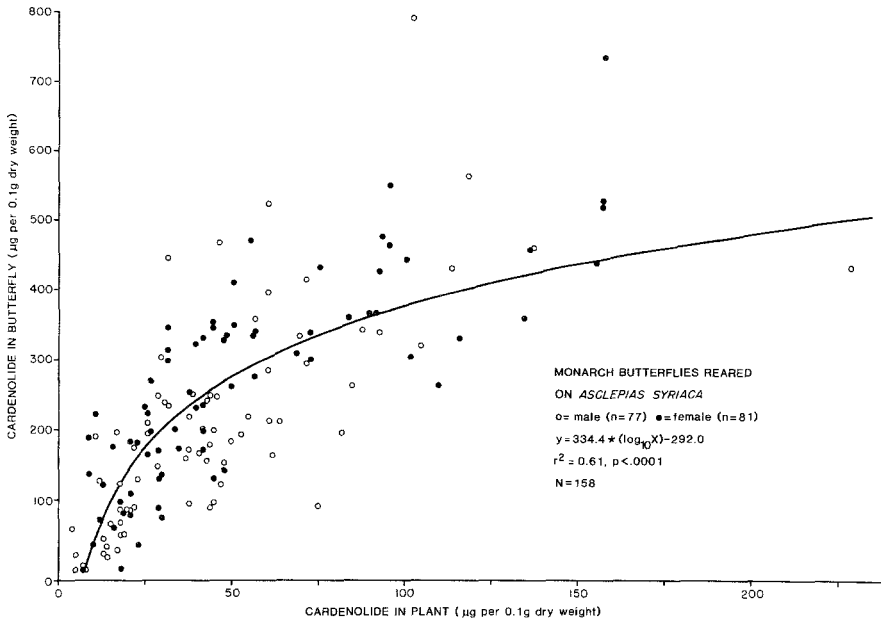


FIG. 3. Cardenolide concentrations of monarch butterflies reared on *A. syriaca* plotted against the cardenolide concentrations of their larval food plants.

and Moffitt, 1974; Cohen, 1985; Lynch and Martin, 1987; Martin and Lynch, 1988). Reasons for this difference between the sexes are considered in the discussion.

As expected, plant concentration remained the most significant determinant of butterfly concentration. However, the area from which the butterfly came had an additional significant main effect on butterfly cardenolide concentration that was independent of the influence of area on plant cardenolide concentration, and there was a significant interaction between plant concentration and area (Table 4B). Consequently, the variation due to location found in the butterfly concentrations is not entirely explained by variation in plant concentration with location, indicating that monarchs in some areas seem able to sequester more cardenolide than monarchs on equivalent plants in other areas. Duncan's multiple-range test split the butterfly sample into eight overlapping groups arranged in practically the same order as the plant samples except for minor reversals (Table 5). Thus the western monarchs from North Dakota (A), Minnesota (B), and upper Michigan (C) had considerably lower cardenolide concentrations than the eastern monarchs from Ontario and the Thumb of Michigan (F), Connecticut (I), and New Jersey, Pennsylvania, and Maryland (J).

TABLE 5. BUTTERFLY CARDENOLIDE CONCENTRATIONS BY REGIONAL AREAS^a

Sample size	Butterfly cardenolide concentration ($\mu\text{g}/0.1\text{ g}$)			Area	Duncan's grouping
	Mean	SD	Range		
12	339	170	128-734	F	1
8	337	114	183-517	I	1
13	313	159	90-564	J	1 2
15	285	122	60-524	H	1 2 3
21	262	146	18-466	G	1 2 3 4
17	251	167	56-792	K	2 3 4 5
20	220	119	7-457	E	3 4 5 6
10	195	149	19-425	L	4 5 6 7
8	170	156	37-470	D	5 6 7 8
15	146	105	0-354	A	6 7 8
8	131	81	0-233	C	7 8
11	99	140	0-380	B	8

^aSee Figure 1 and Table 1. Areas with the same numbers are not significantly different by Duncan's multiple-range test at $P < 0.05$.

Total Cardenolide Content of Butterflies

Since the total cardenolide content of butterflies is the product of cardenolide concentration multiplied by dry weight, we investigated variation in butterfly weight and size (the data are summarized in Table 6). Analyses of covariance of butterfly dry weights and wing lengths against plant cardenolide concentrations, area, sex, and instar collected indicated no growth-related detoxification cost associated with more toxic plants ($F_{1,157} = 2.03$, $P = 0.16$). In addition, there were no significant differences between sexes in their weights ($F_{1,157} = 1.58$, $P = 0.21$) or wing lengths ($F_{1,152} = 0.73$, $P = 0.39$). This is contrary to most earlier findings in which males were heavier than females (Brower et al., 1982; 1984a,b), although Lynch and Martin (1987) report a similar nonsignificant result for monarchs fed *A. viridis*. Only the instar at which butterflies were collected affected their dry weight ($F_{5,157} = 5.71$, $P = 0.0001$) and wing size ($F_{5,152} = 2.88$, $P = 0.0168$). Larvae that were collected at fifth instar produced significantly heavier dry weight adults than those collected in earlier instars ($\bar{X} = 0.19\text{ g}$, $\text{SD} = 0.03$ and $\bar{X} = 0.16\text{ g}$, $\text{SD} = 0.02$, respectively).

Total cardenolide per butterfly (Table 6) was normally distributed and ranged from 0 to 1480 μg (mean = 415 μg , $\text{SD} = 279$). The overall regression, pooling sex, instar, and geographic region, indicates that the total cardenolide per butterfly is significantly dependent on the plant cardenolide concentration

TABLE 6. SUMMARY OF DRY WEIGHTS, TOTAL CARDENOLIDE, CARDENOLIDE CONCENTRATIONS, AND RIGHT FOREWING LENGTHS OF ADULT MONARCHS POOLED OVER ALL AREAS

	Males	Females	Both
Dry weights (mg)			
Mean	0.173	0.178	0.176
SD	0.03	0.03	0.03
Range	0.10-0.24	0.07-0.23	0.07-0.24
N	77	81	158
Total cardenolide (μg)			
Mean	354	472	415
SD	258	287	279
Range	0-1021	0-1480	0-1480
N	77	81	158
Cardenolide concentrations ($\mu\text{g}/0.1$ g dry weight)			
Mean	204	262	234
SD	151	145	150
Range	0-792	0-734	0-792
N	77	81	158
Right forewing lengths (mm)			
Mean	50.8	51.2	51.0
SD	2.3	2.7	2.5
Range	42.0-54.9	40.6-55.5	40.6-55.5
N	74	79	153

($F_{1,157} = 211$, $P = 0.0001$). Since butterfly weight and size were unaffected by area or sex, the analysis of total cardenolide per butterfly mirrors that of cardenolide concentration. Hence analysis of covariance showed that area had a significant direct effect on total butterfly cardenolide content ($F_{11,157} = 2.75$, $P = 0.003$) that was in addition to its influence on plant cardenolide concentration (as was described for butterfly cardenolide concentration above), and there was a significant plant concentration \times area effect ($F_{11,157} = 3.49$, $P = 0.0003$). Once again butterflies from Ontario and the Thumb of Michigan (F), Connecticut (I), New Jersey, Pennsylvania, and Maryland (J) had higher mean total cardenolide contents (Table 7) than those from western areas (North Dakota, Minnesota, and upper Michigan, areas A, B, and C, respectively).

Although adult weight was affected by the larval stage at collection, this did not result in a significant difference in the total butterfly cardenolide content ($F_{5,157} = 0.49$, $P = 0.8$). Thus, although captive early instar larvae were probably given insufficient food, this did not significantly affect adult cardenolide concentrations.

TABLE 7. TOTAL BUTTERFLY CARDENOLIDE CONTENT BY REGIONAL AREAS^a

Sample size	Total cardenolide content (μg)			Area	Duncan's grouping
	Mean	SD	Range		
12	675	351	278-1480	F	1
8	654	270	296-1160	I	1
13	564	305	143-1192	J	1 2
15	533	247	118-1021	H	1 2 3
21	428	245	26-793	G	2 3 4
17	416	222	104-943	K	3 4
20	398	218	13-901	E	3 4
10	349	262	36-734	L	4 5
8	297	315	74-916	D	4 5 6
15	237	162	0-551	A	5 6
8	218	140	0-427	C	5 6
11	153	140	0-380	B	6

^aSee Figure 1 and Table 1. Areas with the same numbers are not significantly different by Duncan's multiple range test at $P < 0.05$.

Despite the similar weight and size of male and female butterflies, females had a significantly higher mean total cardenolide content than males (Table 6; $F_{1,157} = 9.22$, $P = 0.003$). Females were more efficient than males at converting plant cardenolide concentration to total butterfly cardenolide content, since our analysis showed slope homogeneity but a significantly lower intercept for the males [female total cardenolide content = $614 (\log_{10} \text{ plant cardenolide concentration}) - 526$, male total cardenolide content = $614 (\log_{10} \text{ plant cardenolide concentration}) - 596$].

Ecological Effects on Plant and Butterfly Cardenolides

To investigate whether plants and butterflies from different ecological environments have significantly different cardenolide concentrations that might cause the observed regional differences in plant and butterfly cardenolide concentrations, we subdivided the original 27 collection sites in five different ways, according to the ecological characteristics shown in Table 1 and described below. We performed separate ANOVAs for each ecological effect because there were too many empty cells to perform a simultaneous multiway analysis. In all the subsequent groupings, plant cardenolide concentration was not significantly determined by the sex or instar of the associated larvae or by the interactions of these factors with the main effect of primary interest.

Plant Patch Size. *A. syriaca* grows in patches of stems, and each patch

usually represents a single clone (Wilbur, 1976). We searched 245 *A. syriaca* patches that ranged in size from 4 to an estimated 25,000 stems, with a mean of 345 (SD = 1608). The density of monarch eggs and larvae in these patches ranged from 0 to 0.357/stem, with a mean of 0.0077/stem ($N = 245$, SD = 0.0304). Both plants and monarchs were strongly skewed towards smaller patch sizes and lower densities, with 87% of plants in patches of 4–500 stems. Of the total sample, 70% of the plant patches had no monarchs at all, 22% had 0.0009–0.02, and 8% had 0.02–0.36 monarchs per stem. There was no correlation between plant patch size and immature monarch density (Kendall rank correlation coefficient, $\tau = 0.017$, $P = 0.35$).

Plants from small patches had higher cardenolide concentrations than those from large patches ($P = 0.04$). Patches of less than 500 plants had a mean plant cardenolide concentration of 46 $\mu\text{g}/0.1$ g, and those with between 500 and 1000 plants had 44 $\mu\text{g}/0.1$ g, whereas patches of more than 1000 plants had a mean value of 33 $\mu\text{g}/0.1$ g cardenolide. We used these values to calculate the expected mean plant cardenolide concentration of each regional area based on the patch sizes that the plants came from. Referring to Table 1, the mean for each region was weighted to reflect the relative contribution of plants from each patch type to the sample. The rank order of the regional sites predicted by patch size showed no association with their geographical rank order in Table 3 (Kendall $\tau = 0.22$, $P = 0.30$). Hence the regional plant cardenolide differences were not simply caused by differences in patch size. Moreover, patch size was not a significant determinant of butterfly cardenolide concentration, either by analysis of variance, $P = 0.31$, or analysis of covariance, $P = 0.82$. Thus the patch-size differences in plant cardenolide concentration were not sufficiently large to remain detectable in the resultant butterflies after their concentration and storage process.

Plant Maturity. The leaves of young flushing plants had a significantly higher cardenolide concentration than did those of mature plants with ripe seed pods (means = 51 $\mu\text{g}/0.1$ g, and 34 $\mu\text{g}/0.1$ g respectively, $P = 0.004$). The rank order of sites based on plant maturity, weighted for subsample size, showed no association with the rank order of regional plant cardenolide concentrations (Kendall $\tau = 0.24$, $P = 0.27$). Butterflies that were collected from young flushing plants had significantly higher cardenolide concentrations than those on mature plants (273 $\mu\text{g}/0.1$ g compared with 217 $\mu\text{g}/0.1$ g, $P = 0.046$). Analysis of covariance indicates that the butterfly differences were adequately explained by the plant cardenolide concentration differences according to the relationship shown in Figure 3. The rank order of sites determined by plant maturity did not correspond with the observed butterfly cardenolide concentration rank order (Kendall $\tau = 0.19$, $P = 0.38$). Thus differences in plant maturity among sites do not explain the observed regional differences in plant and butterfly cardenolide concentrations.

Plant Habitat. There was a marginally significant difference in cardenolide concentrations of plants from open, shaded, and edge habitats ($P = 0.05$). Duncan's multiple-range comparison showed that the cardenolide content of plants was significantly higher at habitat edges ($49 \mu\text{g}/0.1 \text{ g}$) than in shaded habitats ($30 \mu\text{g}/0.1 \text{ g}$), while the open habitat cardenolide concentration lay between these two values ($39 \mu\text{g}/0.1 \text{ g}$). The rank order of sites by plant habitat did not conform to the regional rank order of sites by plant cardenolide concentration (Kendall $\tau = 0.29$, $P = 0.19$). Differences in butterfly cardenolide concentration between shaded, open, and edge habitats were marginally insignificant by analysis of variance ($P = 0.058$), but Duncan's multiple-range test indicated that at the 5% level butterflies from habitat edges had a significantly higher mean value ($267 \mu\text{g}/0.1 \text{ g}$) than those from shaded habitats ($180 \mu\text{g}/0.1 \text{ g}$), with open habitats intermediate ($242 \mu\text{g}/0.1 \text{ g}$), i.e., similar differences to those of the plants. However, analysis of covariance indicated the butterfly differences were not entirely explained by the plant differences, but differences between habitats directly affected the butterflies ($P = 0.015$). There was no significant association between the expected rank order of sites based on their habitat characteristics and the observed butterfly cardenolide concentration order (Kendall $\tau = 0.29$, $P = 0.19$). Hence these habitat differences do not explain the regional plant and butterfly cardenolide concentration differences.

Other Ecological Effects. There were no significant differences ($P = 0.24$) among plant cardenolide concentrations when subdivided on the basis of site topography into flat sites ($36 \mu\text{g}/0.1 \text{ g}$), Great Lakes sites ($37 \mu\text{g}/0.1 \text{ g}$), or hilly sites ($46 \mu\text{g}/0.1 \text{ g}$). Similarly, there was no significant difference between roadside ($39 \mu\text{g}/0.1 \text{ g}$) and pasture ($39 \mu\text{g}/0.1 \text{ g}$) populations ($P = 0.99$). None of these factors had significant effects on butterfly cardenolide concentration.

TLC Cardenolide Fingerprint of Plants and Butterflies

The means and standard deviations of the R_d , spot intensity (SI) and probability of occurrence (PO) values for all visualized cardenolide spots in the 146 paired plant and butterfly samples are given in Table 8, and their overall pattern is illustrated in Figures 4 and 5 (12 of the original 158 paired samples had insufficient cardenolide to produce a cardenolide fingerprint). Several spots were frequently weak or absent in plants and butterflies but the standard deviations of the R_d values of all spots were low, once again indicating consistent separation and resolution of spots (cf. Brower et al., 1984a).

A total of 28 cardenolides were identified, of which 27 appeared in the plants and 21 in the butterflies. Spot 20, which corresponded with the mobility of the desglucosyrioxide standard, did not appear in any of the plants, and spots 19, 21, and 24–28 did not appear in any butterflies. Thus there were 20 spots in common. The migration distance of the digitoxigenin standard is based on

TABLE 8. MEANS AND STANDARD DEVIATIONS FOR R_D AND SI VALUES FOR 28 CARDENOLIDES, WITH THEIR PROBABILITIES OF OCCURRENCE (PO), IN 146 *A. syriaca*-MONARCH BUTTERFLY PAIRED SAMPLES

Spot number	Parameter	Plant			Butterfly		
		Mean	SD	PO	Mean	SD	PO
28	R_d	3.58	0.60	0.03			
	SI	1.25	0.50				
27	R_d	3.16	0.39	0.39			
	SI	1.56	0.68				
26	R_d	2.79	0.32	0.33			
	SI	1.15	0.36				
25	R_d	2.54	0.28	0.61			
	SI	1.30	0.53				
24	R_d	2.28	0.38	0.25			
	SI	1.11	0.32				
23	R_d	1.73	0.31	0.03	1.64	0.04	0.06
	SI	1.00	0.00		1.33	0.71	
22	R_d	1.44		0.01	1.42	0.10	0.48
	SI	1.00			1.56	0.67	
21	R_d	1.35	0.10	0.36			
	SI	1.71	0.72				
20	R_d				1.26	0.08	0.70
	SI				2.19	0.99	
19	R_d	1.16	0.09	0.66			
	SI	1.86	0.61				
18	R_d	1.06	0.06	0.38	1.04	0.07	0.08
	SI	1.79	0.68		1.36	0.50	
17	R_d	1.00	0.02	0.78	0.98	0.03	0.82
	SI	1.82	0.65		1.73	0.77	
16	R_d	0.96	0.03	0.52	0.95	0.02	0.84
	SI	1.82	0.67		2.15	0.94	
15	R_d	0.90	0.04	0.57	0.89	0.03	0.80
	SI	1.37	0.56		2.04	0.94	
14	R_d	0.86	0.04	0.37	0.86	0.03	0.71
	SI	1.35	0.59		2.03	0.94	
13	R_d	0.79	0.04	0.73	0.79	0.04	0.84
	SI	1.64	0.65		2.31	0.83	
12	R_d	0.75	0.04	0.53	0.76	0.04	0.88
	SI	1.52	0.60		2.28	0.87	
11	R_d	0.72	0.05	0.25	0.71	0.03	0.38
	SI	1.43	0.60		2.04	0.76	
10	R_d	0.67	0.05	0.14	0.68	0.04	0.27
	SI	1.30	0.47		1.69	0.77	
9	R_d	0.58	0.05	0.62	0.58	0.04	0.82
	SI	1.62	0.79		2.75	0.98	
8	R_d	0.48	0.04	0.99	0.47	0.03	1.00
	SI	3.04	0.93		4.78	0.54	

TABLE 8. Continued

Spot number	Parameter	Plant			Butterfly		
		Mean	SD	PO	Mean	SD	PO
7	R_d	0.38	0.04	0.18	0.38	0.03	0.70
	SI	1.54	0.81		1.95	0.92	
6	R_d	0.33	0.04	0.59	0.34	0.03	0.92
	SI	1.53	0.65		2.83	0.84	
5	R_d	0.32	0.03	0.30	0.30	0.03	0.92
	SI	1.50	0.59		2.79	0.80	
4	R_d	0.26	0.04	0.28	0.24	0.03	0.90
	SI	1.12	0.33		2.62	0.74	
3	R_d	0.23	0.03	0.14	0.22	0.03	0.87
	SI	1.00	0.00		2.54	0.82	
2	R_d	0.09	0.03	0.34	0.10	0.03	0.71
	SI	1.18	0.44		1.30	0.54	
1	R_d	0.02	0.02	0.31	0.04	0.04	0.41
	SI	1.24	0.43		1.33	0.54	

81 spotted samples (three per 27 plates), giving an R_d value of 2.75 (SD = 0.31). Spot 8, the most concentrated and characteristic cardenolide present in both *A. syriaca* plants and the monarchs that fed on them (see Figures 4 and 5), always corresponded with the aspecioside standard. By inference with published data on the mobilities of known cardenolides in the same TLC solvent system, spots 3 and 4 are likely to be syrioxide and uzarin, respectively, spot 9 is syriobioside, 16 is probably syriogenin, 24 or 25 is uzarigenin, 26 may be labriformidin, and 27 may be labriformin (Nelson et al., 1981; Seiber et al., 1983, 1986; Brower et al., 1984a).

Plants. Of the 27 spots, 17 were often absent, resulting in PO s of less than 0.40 (Table 8). This left 10 diagnostic spots, seven below digitoxin, one at this level, and two between digitoxin and digitoxigenin. Seventeen of the 27 plant spots (63%) occurred between the origin and level of digitoxin, seven (26%) between digitoxin and digitoxigenin, and three (11%) above digitoxigenin.

Butterflies. All the butterfly spots had mean SI values greater than 1.25, and they were generally greater than their corresponding plant SI values (Table 8). This probably accounts for the higher PO values of the butterfly spots (Table 8); 17 of the 21 butterfly spots occurred with a $PO > 40\%$. Seventeen spots (81%) occurred below digitoxin, and four (19%) occurred between the digitoxin and digitoxigenin levels. No butterfly had cardenolides with R_d values above digitoxigenin. The butterflies stored cardenolides within the more polar 46% of the plant R_d range (R_d spot 23/ R_d spot 28).

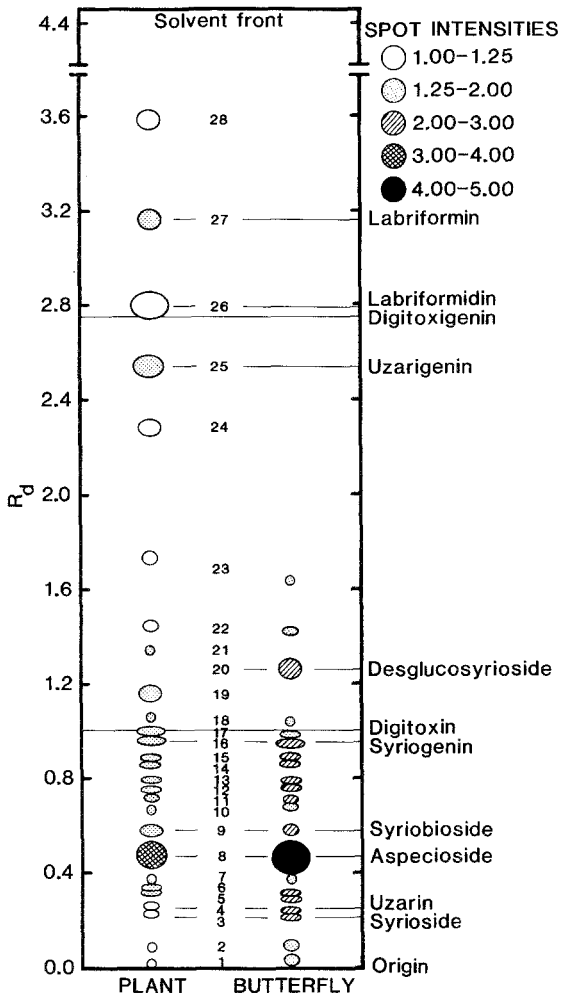


FIG. 4. TLC cardenolide patterns, in the chloroform-methanol-formamide (90:6:1) solvent system at 20°C, of monarch butterflies and their *A. syriaca* larval host plants, drawn from plotted R_f and SI values of TNDP-positive spots. The spot sizes of the 28 cardenolides are drawn to correspond with those on photographs of the original 27 TLC plates (see Figure 5). The four cardenolide standards (digitoxin, digitoxigenin, aspecioside, and desglucosyrioside) were spotted on each plate (see Figure 5). Remaining cardenolide identities (syrioside, uzarin, syriobioside, syriogenin, uzarigenin, labriformidin, and labriformin) were inferred from their published R_f values in the same solvent system.

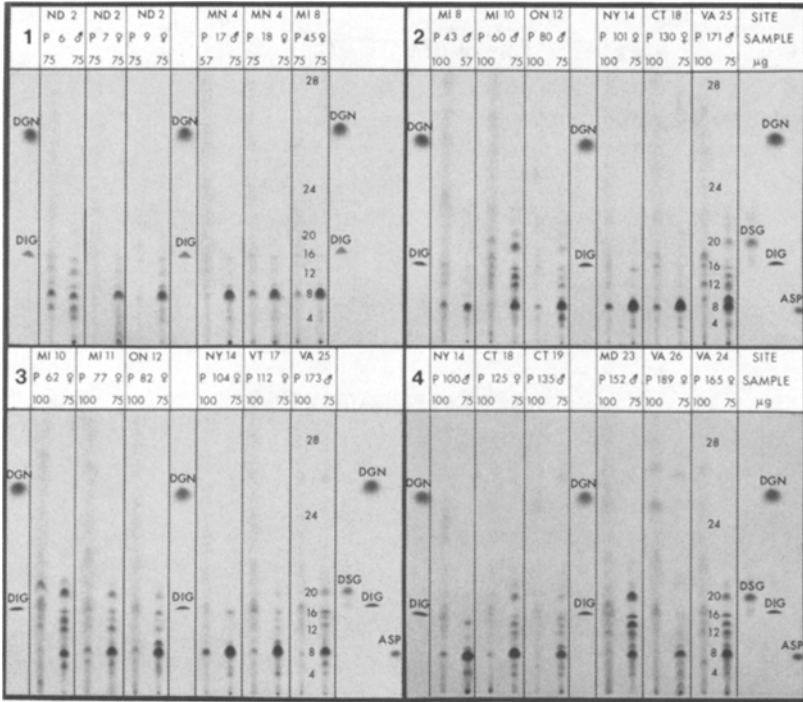


FIG. 5. Photographs of four TLC plates showing the fingerprint pattern of TNDP-visualized cardenolide spots in extracts of monarch butterflies and their *A. syriaca* host plants, separated in chloroform-methanol-formamide (90:6:1) at 20°C, from North Dakota east to Vermont and south to Virginia. Each plate shows six plants and their corresponding monarch butterflies. The four cardenolide standards run on the plates are: ASP = aspecioside; DIG = digitoxin; DGN = digitoxigenin; DSG = desglucosyrioside. Each plant-butterfly channel pair is labeled with the state, site number (see Figure 1 and Table 1), plant (P), butterfly sex, sample pair number, and the respective amount of cardenolide spotted (μg , equivalent to digitoxin). Plant channels showing weak resolution resulted from pigment interference in the original spectroassay (see text). Spots 4, 12, 16, 24, and 28 correspond to those in Figure 4. Spots 8 and 20 always coincided with the mobilities of the cochromatographed standards aspecioside and desglucosyrioside, respectively.

Variation in Plant R_d and SI Values. Plant R_d values were significantly influenced by the regional area from which they derived ($F_{11,1557} = 2.13$, $P = 0.016$) and the TLC plate on which they were run ($F_{26,1557} = 1.72$, $P = 0.013$), but they showed no significant association with larval sex ($F_{1,1557} = 0.64$, $P = 0.42$) or any interactions of these main effects. We investigated whether this

R_d variation resulted from particular spot occurrences (and hence patterns) being peculiar to specific areas using a 12 (areas) by 27 (spots) contingency table, with a similar analysis for the plates (27 plates \times 27 spots). We found very high uniformity among the different treatments, spots having the same POs across areas ($\chi^2 = 241$, $df = 286$, $P = 0.98$) and across plates ($\chi^2 = 670$, $df = 676$, $P = 0.56$). This indicates that the variation in plant R_d values does not reflect significant pattern differences among areas or plates, but is the cumulative result of variation around the mean R_d value of each spot.

Plant spot intensity values varied with area ($F_{11,1557} = 3.49$, $P = 0.0001$), but not with plate ($F_{26,1557} = 1.34$, $P = 0.12$), the sex of the larva associated with the plant ($F_{1,1557} = 1.31$, $P = 0.25$), or any interactions of these variables. Generally, the more northern and western samples had more intense spots than the more southern and eastern ones.

Plant-Butterfly Spot Relationships: Effects of Area, Sex, and Plate on R_d and SI Values. We used analysis of covariance to test simultaneously for the dependence of the R_d and SI values of all the butterfly spots on their respective plant spots and for the effects of the 12 areas, butterfly sex, the 27 plates, and interactions between these terms. The analysis could only use observations where both plant and butterfly spots were represented, i.e., 981 of the possible 2920 (146 \times 20) spots in common.

The butterfly R_d values were strongly dependent on those of the plant ($F_{1,980} = 50111$, $P = 0.0001$). The simple regression model that examined the dependency of butterfly R_d on plant R_d alone showed a correlation of $r^2 = 0.98$. (The TLC spot comparison methodology largely predetermines the correspondence of the respective plant and butterfly spots.) The full model improved the fit slightly ($F_{174,980} = 597.76$, $P < 0.0001$, $r^2 = 0.99$) and indicated significant area ($F_{11,980} = 7.70$, $P = 0.0001$) and plate ($F_{26,980} = 11.48$, $P = 0.0001$) effects with significant interactions between plant R_d and area ($F_{11,980} = 6.25$, $P = 0.0001$), plant R_d and plate ($F_{26,980} = 12.83$, $P = 0.0001$), and plant R_d and sex ($F_{1,980} = 3.91$, $P = 0.05$), although butterfly sex was not a significant main effect ($F_{1,980} = 2.14$, $P = 0.14$). Although this analysis indicates significant variation in butterfly R_d , contingency table analyses of spot by area and of spot by plate showed remarkable uniformity in spot occurrences over the different locations ($\chi^2 = 120$, $df = 220$, $P = 1.0$) and plates ($\chi^2 = 291$, $df = 520$, $P = 1.0$), indicating a consistent *A. syriaca* fingerprint pattern. Rather than substantial pattern differences, it appears that the variation in butterfly R_d reflects variation around the mean R_d of each spot. The information in Table 8 indicates these differences are very small, but they are detectable because so much of the variation in butterfly R_d is explained by variation in plant R_d .

There was a weak, but significant, correspondence of butterfly SI values with plant SI s ($F_{1,980} = 400.90$, $P = 0.0001$). The main effects, plate ($F_{26,980}$

= 2.28, $P = 0.0003$) and area ($F_{11,980} = 3.30$, $P = 0.0002$), also had significant effects on butterfly *SIs* but the butterfly sex did not ($F_{1,980} = 0.25$, $P = 0.62$), and none of the interactions were significant. There was substantial overlap in *SI* values over the different areas, with no consistent pattern of division into clearly defined geographical zones.

DISCUSSION

Late Summer Breeding Distribution of Monarch Butterflies

Our field sampling corroborates previous unsubstantiated assumptions that by late summer monarchs breed across the entire range of *A. syriaca*. Since we found this milkweed growing abundantly in old fields, pastures, disturbed areas, and along roadsides and railways across the entire sampled range in August, and found no other milkweed host species approaching this range and abundance, it is probable that *A. syriaca* is in fact the principal milkweed host of overwintering monarchs that begin their southward autumn migration to Mexico in September (Urquhart and Urquhart, 1978; Brower, 1985). Other widely distributed milkweed species, like *A. incarnata*, *A. verticillata*, and *A. tuberosa* (Woodson, 1954), may also be used (Malcolm and Brower, 1986), but it is unlikely that they approach the significance of *A. syriaca* in the life history of monarch butterflies.

Quantitative Cardenolide Storage

Plants. We found that the mean cardenolide concentration of *A. syriaca* leaves is low, but, like other *Asclepias* species, there is considerable variation among plants. Previous investigations of *A. syriaca* leaf material cite low cardenolide concentrations, from immeasurable to 264 $\mu\text{g}/0.1\text{ g}$ (similar to our range of 4–229 $\mu\text{g}/0.1\text{ g}$), and a 40-fold variation among 16 samples from one site (Feir and Suen, 1971; Roeske et al., 1976; Isman et al., 1977; Nelson et al., 1981; Seiber et al., 1983). In-depth studies have yet to establish the ranges of cardenolides in the other widespread, eastern North American milkweeds recorded as monarch food plants, including *A. verticillata*, *A. tuberosa*, *A. incarnata*, *A. viridiflora*, *A. hirtella*, *A. exaltata*, *A. variegata*, *A. purpurascens*, and *A. amplexicaulis* (Malcolm and Brower, 1986), but preliminary evidence suggests that all these plants are at least as low, or lower in cardenolide than *A. syriaca* (Roeske et al., 1976; Seiber et al., 1983). Of the eight *Asclepias* species that have been analyzed in a similar manner to the present study, the variation in cardenolide concentration of *A. syriaca* is most like *A. speciosa* (Brower et al., 1984a), *A. californica* (Brower et al., 1984b), and *A. cordifolia* (Brower et al., in preparation). All four species have low plant cardenolide

concentrations with positively skewed distributions, such that most plants contain very little cardenolide.

We found plants on the eastern side of *A. syriaca*'s range to have a higher cardenolide concentration than those in the west. There was an almost fivefold difference in mean plant cardenolide concentration across the 12 areas we sampled. This compares with an approximate threefold difference among the California populations of *A. speciosa* (Brower et al., 1984a) and *A. californica* (Brower et al., 1984b), and an approximately twofold difference for *A. cordifolia* (Brower et al., in preparation). The *A. syriaca* variation is greater, but we sampled a much wider area both latitudinally and longitudinally, and the total sample size was larger. Because the variation in plant cardenolide concentration appears continuous, with no obvious regional discontinuities, we do not think it suggests different *A. syriaca* races. Instead, the high regional variance in cardenolide content of *A. syriaca* may simply be a characteristic feature of those *Asclepias* species with low mean levels of cardenolide.

Following Rosenthal and Janzen (1979), Brower et al. (1982) suggested that, aside from genetic factors, a host of environmental factors, such as temperature, altitude, nutrient availability, plant competition, and herbivore pressure may influence the production of defensive secondary chemicals in plants. Any or all of these might result in the regional variation we report. Thus we grouped our data in five different ways that we thought might reflect how such environmental factors could be acting on the plant sample.

Our analyses show a substantial north-south element to the east-west regional difference. Nelson et al. (1981) and Brower et al. (1982) suggested that the north-to-south increase in the cardenolide concentrations of their *A. eriocarpa* samples was a temporal rather than geographic phenomenon since the sample with the highest concentration was collected in June, while the lowest was collected in September. However, difference in sampling date is unlikely to explain the geographic variability of *A. syriaca*, since our total sampling period spanned only four weeks compared with their four months. More significantly, as in Nelson et al. (1981) and Brower et al. (1982), our data indicate independently that plants have higher cardenolide concentrations when they are younger than when older. Hence, we would expect the western plants to be *more* concentrated than the eastern plants, rather than the reverse as we found, because the western *A. syriaca* plants were not only sampled earlier in the season but were also younger by virtue of their more northerly location. The geographical differences we have found thus appear robust.

Young flushing plants had higher cardenolide concentrations than mature individuals, and plants in small patches had higher concentrations than those in large patches. In both cases this may reflect lower production or degradation of cardenolides in older plants (assuming that large clones have been established for longer than small ones). Overall, quantitative plant cardenolide defenses

appeared most in evidence in young, flushing, prereproductive individuals, and along habitat edges. However, none of these ecological groups accounted for the observed regional difference in plant cardenolide, and the ecological variation was never as great as the regional variation.

Abundant milkweeds, like *A. syriaca*, with high variance, low mean and skewed cardenolide distributions may lower the impact of specialist herbivory by reducing the availability of defensive chemicals to aposematic herbivores, like the monarch, for use in their own chemical defenses against predators. Thus we speculate that *A. syriaca* may be at a threshold of reduced specialist herbivore fitness and enhanced ability of generalist herbivores to exploit the weakly defended plant resource. The east-west gradient of cardenolide content could then be the result of higher generalist herbivory in the east and higher specialist herbivory further west by insects like the monarch.

Butterflies. The monarchs stored cardenolides more efficiently from plants of low, than from plants of high, concentrations. Similar logarithmic relationships occur in monarchs reared on *A. speciosa* (Brower et al., 1984a), *A. californica* (Brower et al., 1984b), and *A. cordifolia* (Brower et al., in preparation; Nelson, 1989), all of which have low mean plant cardenolide concentrations of less than 100 $\mu\text{g}/0.1\text{ g}$, and also for the southern *A. viridis* with 245 $\mu\text{g}/0.1\text{ g}$ cardenolide (Lynch and Martin, 1987). Indeed, it seems likely that a logarithmic curve applies to the uptake and storage of cardenolide from all milkweed species. However, in individual plants or species that have high concentrations, the butterfly concentration becomes constant because it reaches storage capacity and is thus operating at the curve's asymptote. Examples include the apparently linear conversion relationships for *A. eriocarpa* (Brower et al., 1982), *A. asperula* (Martin and Lynch, 1988), *A. vestita* and *A. erosa* (Brower et al., in preparation), all of which have mean plant cardenolide concentrations above 400 $\mu\text{g}/0.1\text{ g}$ (Nelson, 1989).

Because most plants had low cardenolide concentrations, logarithmic storage by monarchs resulted in considerably higher mean butterfly, than mean plant, concentrations, and an expanded range of butterfly concentrations. These trends are again consistent with those of other milkweeds with low cardenolide concentrations, although the *A. syriaca* data show the widest range and some of the highest concentrations of milkweed-derived cardenolide storage by monarch butterflies reported to date.

Although a logarithmic uptake curve is a good model of cardenolide storage across a range of milkweed species, butterflies were more efficient at storing cardenolides from *A. syriaca* than they were from *A. speciosa*, *A. californica*, or *A. cordifolia*. Nelson (1989) suggests that the storage of cardenolides from mixtures by larvae is, at least in part, additive. Since cardenolide storage is affected by cardenolide structure, the structure of *A. syriaca* cardenolides must facilitate storage by monarchs (Seiber et al., 1980; Nelson, 1989). For exam-

ple, the nonpolar plant cardenolide labriformin is readily converted by monarch larvae to the more polar desglucosyrioside, and cardenolides with doubly linked sugars, or those with C-3 hydroxyls, appear prevalent in *Asclepias* species and are selectively stored by monarch butterflies (Seiber et al., 1980; Nelson et al., 1981; Brower et al., 1982, 1984a; Nelson, 1989). Perhaps this indicates a special evolutionary relationship between the monarch butterfly and *A. syriaca*, in which the wide range and abundance of *A. syriaca* has resulted in strong selection for efficient storage of *A. syriaca* cardenolides to enhance the monarch's chemical defense. Indeed, if we are correct in speculating that the low cardenolide content of *A. syriaca* is a selected response to monarch herbivory, which reduces the effectiveness of monarch chemical defenses, then efficient cardenolide storage by monarchs on *A. syriaca* may indicate some reciprocity in their diffuse coevolution.

Female monarchs had significantly higher cardenolide concentrations than males, in agreement with prior studies. Previously, where females were significantly smaller than males (Brower et al., 1982, 1984a,b) this might have resulted simply from the higher surface area to body volume ratio of females since cardenolides are stored primarily in the exoskeleton (Parsons, 1965; Brower and Glazier, 1975; Nishio, 1980; Brower et al., 1988). However, in the present study, where there was no significant size difference between the sexes, the data suggest that females are more efficient at cardenolide storage than males. Hence females from *A. syriaca* also had significantly more total cardenolide than males, while earlier studies found no significant difference in total cardenolide content between the sexes, where it was suggested that the smaller female size canceled out the effect of higher female cardenolide concentrations.

The ability of butterflies to store cardenolides logarithmically from *A. syriaca* compensated for the relatively small differences among milkweeds from different sized plant patches, so there were no discernible differences among the monarchs that derived from them. However, there were significant differences in the cardenolide concentrations of butterflies from plants of differing maturity, from edge compared with shaded habitats, and from different collection areas. These butterfly differences were not entirely explained by the differences in plant cardenolide concentration, but they indicate a direct effect on the butterflies themselves. In particular, the cardenolide concentrations of butterflies from the Ontario region were higher than expected on the basis of plant cardenolide concentration, while those of butterflies from northern Virginia were lower than expected, although area still significantly effected butterfly cardenolide concentration even when these extreme cases were removed from the analysis. Brower et al. (1982) suggested that abiotic environmental influences could produce such an effect. For example, slower larval development at cooler temperatures might allow more time for cardenolide accumulation and result in

higher cardenolide butterflies in shaded conditions, or from a colder, more northerly area such as Ontario, compared with a warmer, more southerly area like Virginia.

Qualitative Cardenolide Storage: A. syriaca TLC Fingerprint

We found a remarkably consistent *A. syriaca* plant fingerprint among all our sites, contrary to earlier reports of variable numbers of cardenolides in different North American and European populations of *A. syriaca* (Brower, 1969; Duffey, 1970; Rothschild et al., 1975; Roeske et al., 1976; Brown et al., 1979; Brower et al., 1982, 1984a; Moore and Scudder, 1985). It seems likely that different methodologies and variable extraction efficiencies in the earlier studies are responsible for these disparities. Moreover, when cardenolide concentration was low, or the original sample weight was small, spots may have been missing from the cardenolide profile when the cardenolide was in fact present. Like us, Duffey and Scudder (1972) and Moore and Scudder (1985) found that the TLC cardenolide patterns of *A. syriaca* leaf and seed samples from different locations were very similar, with only a minor difference in seeds from Connecticut. The geographical variability in quantitative cardenolide storage that we describe above is probably produced by quantitative variation in the amounts of individual cardenolides. Thus area significantly influenced cardenolide spot intensities but did not result in qualitative differences in the presence or absence of particular cardenolides within the entire fingerprint.

Given the high consistency of the plant's cardenolide fingerprint, together with the monarch's logarithmic storage capacity, it is not surprising that the butterfly cardenolide fingerprint was also remarkably consistent across the sampled range of *A. syriaca*. The most diagnostic cardenolide of the *A. syriaca* monarch fingerprint is spot 8, corresponding with the cardenolide aspecioside. Aspecioside is also the most concentrated cardenolide in the plant, but even so, monarchs increase its relative concentration. Large amounts of aspecioside may accumulate slowly during larval feeding because monarch Na^+, K^+ -ATPase is insensitive to polar cardenolides (Vaughan and Jungreis, 1977), and the low permeability of tissues to polar cardenolides may make their storage in the monarch's exoskeleton (Brower and Glazier, 1975; Nishio, 1980) physically simpler than more readily mobilized nonpolar cardenolides (Yoder et al., 1976; Duffey, 1977; Duffey et al., 1978; Scudder and Meredith, 1982). The presence of large amounts of polar cardenolide may also explain the low emeticity of *A. syriaca*-derived monarchs, as discussed below.

Estimation of Emetic Toxicity of Monarchs Reared on A. syriaca

Brower and Moffitt (1974) determined the 50% blue jay emetic dose (ED_{50}) of monarch butterflies captured during their autumn migration through western Massachusetts in 1971, and from their data Fink and Brower (1981) calculated

the ED₅₀ as 398 μg of cardenolide/85 g bird (Table 9). Fingerprints of 24 of these same butterflies proved uniform (Roeske et al., 1976) and are consistent with the *A. syriaca* pattern described in the present study. Given the extensive breeding on *A. syriaca* by the late summer generation of monarchs in north-eastern America, together with these fingerprint determinations and the paucity of other *Asclepias* species at this time of year, it is virtually certain that these butterflies had fed on *A. syriaca*.

In an attempt to verify this ED₅₀ value of the wild-collected autumn migrants, a group of monarchs was reared in the laboratory on wild-collected *A. syriaca* plants from western Massachusetts in August 1969 (L.P. Brower and S.C. Glazier, unpublished data). It was found that 83 monarchs had a mean cardenolide concentration of 278 $\mu\text{g}/0.1$ g (SD = 76; range = 108–453; males, $N = 37$, mean = 263, SD = 76, range = 108–402; females, $N = 46$, mean = 291, SD = 74, range = 137–453). Note that these monarchs had cardenolide concentrations very similar to our field-collected monarchs, particularly those from New York and Vermont (areas G and H, Figure 2; Table 5).

After spectroassay of these *A. syriaca* monarchs, 19 blue jays were force fed between 0.111 and 0.257 g of the dry, powdered butterfly material. Two of the jays vomited when given 395 and 581 μg cardenolide/85 g bird. However, contrary to expectation, none of the nine birds given more cardenolide (587–1021 $\mu\text{g}/85$ g bird) vomited, and none of the eight birds given less cardenolide (261–564 $\mu\text{g}/85$ g bird) vomited. The failure of these freshly hatched *A. syriaca* monarchs to elicit emesis at cardenolide doses exceeding 1000 $\mu\text{g}/85$ g bird, compared to a clear ED₅₀ of 398 $\mu\text{g}/85$ g bird, for the *A. syriaca*

TABLE 9. TOTAL CARDENOLIDE CONTENTS AND ESTIMATES OF EMETIC POTENCIES OF MONARCH BUTTERFLIES PRESUMED OR KNOWN TO HAVE FED ON *A. syriaca* IN NORTHEASTERN U.S.A.

Sample	Date	N	Total cardenolide (μg)		ED ₅₀ ^a	Source ^b
			Mean	Range		
Autumn migrants, MA	Sept. 1971	355	384	34–850	398	1, 2
Overwintering, Mexico	Jan. 1980	198	128	0–551	323	2
Wild reared, ND-VA	Aug. 1983	158	472	0–1480	—	3
Laboratory reared, MA	Aug. 1969	83	455	182–790	>1000	4

^a $\mu\text{g}/85$ g blue jay

^b 1, Brower and Moffitt (1974); 2, Fink and Brower (1981); 3, this paper; 4, L.P. Brower and S.C. Glazier, unpublished data.

butterflies from the wild requires explanation. One possibility is that the chemical structures of the sequestered cardenolides change in the butterflies as they age in a way which increases their emetic toxicity. In support of this possibility, Nishio (1980) observed chemical modification as well as shifts in the proportion of polar and nonpolar cardenolides during the development of *A. humistrata*-reared monarchs. Such a postulated process in aging monarchs may also be accompanied by gradual cardenolide excretion or catabolism. Evidence for this is the fact that the mean total cardenolide of overwintering monarchs in Mexico (128 μg) is lower than the means for monarchs reared from all 12 of our sites and for both autumn migrants and laboratory-reared monarchs in Massachusetts (Tables 7 and 9).

The cardenolide storage pattern of *A. syriaca* butterflies is extremely similar to that of *A. speciosa* and, like it, has a preponderance of more polar cardenolides (Brower et al., 1984a). We therefore should expect monarchs reared on the two species to have a comparable blue jay ED_{50} assay. However, the ED_{50} of *A. speciosa* monarchs was only 192 μg cardenolide/85 g bird (from Brower et al., 1984a), i.e., their emetic toxicity was about twice that of the migrant *A. syriaca* monarchs ($\text{ED}_{50} = 398 \mu\text{g}/85 \text{ g bird}$, Table 9). We hypothesize that this lower emeticity is based on quantitative differences in proportions of the shared cardenolides of their two very similar TLC patterns (Brower et al., 1984a; Seiber et al., 1986). Since lower, compared to higher, polarity cardenolides can cross epithelial tissues more quickly (Wright, 1960; Duffey and Scudder, 1974; Yoder et al., 1976; Duffey, 1977; Duffey et al., 1978; Scudder and Meredith, 1982), the higher concentrations of lower polarity cardenolides, such as desglucosyrioxide in *A. speciosa*-reared monarchs, may trigger stronger emetic contractions in smooth muscles of a vertebrate predator's gut than extracts of *A. syriaca*-reared monarchs.

Based on the above findings, it is reasonable to consider 323–398 μg cardenolide as the approximate ED_{50} value for autumn migrant and overwintering monarchs of the eastern population. Hence butterflies from different regions would be expected to have different degrees of protection. In fact, four of our 12 collection areas (Table 7) produced butterflies that, on average, are substantially below the required ED_{50} dose. These are the western group, including North Dakota, Minnesota, and western Michigan.

Implications of A. syriaca Fingerprint for Understanding Annual Cycle of Monarch Butterflies

What are the implications of this variation in cardenolide content and emetic potency for the contribution of *A. syriaca* to the pool of Mexican overwintering butterflies? Wild Massachusetts monarchs (Table 9) have a similar, but narrower total cardenolide range of 34–850 μg , compared with our *A. syriaca*-

derived monarchs' range of 0–1480 μg and, like ours, it is normally distributed, although with a lower mean of 384 μg , compared to 472 μg . Yet both of these distributions differ considerably from the Mexican sample (Table 9), which has a similar ED_{50} value to the Massachusetts sample but has a positively skewed cardenolide distribution with a greater proportion of less protected, low cardenolide butterflies. This shift towards lower cardenolide values with time is contrary to expectations if predators take less protected butterflies (Fink and Brower, 1981; Brower et al., 1985). It seems highly unlikely that the earlier Massachusetts sample contains considerable numbers of toxic southern butterflies, as suggested by Martin and Lynch (1988), since it was not collected until September (Brower and Moffitt, 1974), by which time we would expect a huge preponderance of *A. syriaca*-derived butterflies resulting from as many as three northern generations (Borkin, 1982; Malcolm et al., 1987), and the butterflies were in mint condition, suggesting recent eclosion (L.P. Brower, personal observations). Furthermore, the variation that Brower and Moffitt (1974) and Fink and Brower (1981) cite for the Massachusetts sample lies within the range we have determined for *A. syriaca*-reared butterflies, and they have the same fingerprint as our *A. syriaca*-reared butterflies (compare the TLC fingerprint patterns of the same wild Massachusetts butterflies in Figure 7 of Roeske et al., 1976, with Figures 4 and 5 of this paper and plate 2D of *A. syriaca* monarch parts in Brower, 1985). Thus there is no need to suggest the influence of non-*syriaca*-derived butterflies.

Nevertheless, the overwintering cardenolide distribution does change with time, and consideration of monarch overwintering biology suggests four possible explanations. First, low cardenolide butterflies could be represented in greater numbers at the Mexican overwintering sites because more monarchs are produced from areas with milkweeds of lower cardenolide content and proportionately more of these butterflies reach Mexico. Second, there may be an aging effect related to energy expenditure in flight, or preferential excretion and metabolism of certain cardenolides over time. Third, there may be greater mortality of higher cardenolide monarchs with time because of a physiological cost of cardenolide storage. Fourth, apparent cardenolide loss through time may be an artifact of differential size mortality, unrelated to cardenolides per se. For example, predators may take larger monarchs, carrying more cardenolide, simply because they provide most food. Further work is needed to explore these hypotheses.

Since *A. syriaca* is the preeminent summer larval host plant of monarch butterflies in eastern North America, and because we have established the consistency of the *A. syriaca* TLC fingerprint, this paper provides the basis to tackle a number of pressing ecological and physiological questions. We will now be able to determine (1) the proportion of autumn migrants that fed on *A. syriaca*; (2) the proportion of Mexican overwintering monarchs that fed on *A.*

syriaca; (3) the temporal change of overwintering monarch cardenolides and its impact on predation and subsequent remigration; (4) whether overwintered monarchs with the *A. syriaca* fingerprint do return north to breeding sites and how far north they migrate; and (5) the proportion and timing of *A. syriaca*-bred monarchs that reach different breeding latitudes in the spring. Thus we will be able to describe the overall temporal and spatial pattern of the monarch's spring remigration. Answers to these questions will address some of the most consequential and topical issues in our understanding of monarch migration and the ecological chemistry of milkweed-monarch-predator interactions.

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INSECTICIDAL ACTIVITY OF HYPERICIN TOWARDS *Manduca sexta* LARVAE

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Abstract—The toxic effect of hypericin (HYP), a photodynamic quinone that occurs in certain species of the genus *Hypericum*, towards *Manduca sexta* third-instar larvae was investigated. The LD₅₀ of purified hypericin was 16 µg/g larval initial fresh weight in constant light (22 W/m²). Reduced irradiance resulted in decreased mortality. Sublethal applications retarded larval growth (body fresh weight) in a dose-dependent manner. Toxicity had an absolute light dependence at the dose levels used, the active wavelengths being >500 nm. The phototoxic effect was rapidly lost when larvae were maintained in darkness (>8 hr) prior to irradiation. However, the potential for light-dependent mortality was retained if larvae were starved prior to irradiation.

Key Words—Hypericin, *Hypericum perforatum*, photodynamic quinone, photosensitizer, singlet oxygen, *Manduca sexta*, Lepidoptera, Sphingidae.

INTRODUCTION

It is increasingly apparent that interactions of species occur via subtle chemical mechanisms. Indeed, the ecological interactions between insects and plants mediated by photodynamic compounds are an important developing field (Towers, 1984; Downum and Rodriguez, 1986).

Hypericin (HYP), a naturally occurring photodynamic compound (Knox and Dodge, 1985a), causes photosensitization of grazing animals following ingestion of certain species of *Hypericum* (Giese, 1980). However, the evolutionary significance of these types of secondary plant compounds appears to be

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related to phytophagous insect-host plant interactions. Photodynamic compounds may constitute a chemical defense against phytophagous insects by acting as feeding deterrents (McLachlan et al., 1982; Champagne et al., 1986). This role for secondary plant compounds has been well documented (e.g., Rosenthal and Janzen, 1979).

Differences in susceptibility to phototoxic compounds may be dependent upon rates of sequestration and metabolism, or cuticle sclerotization and depositions of melanin in the cuticle, i.e., a "black" mutant strain of *Manduca sexta* larva is more resistant to the effects of photosensitizers than normally pigmented larva (Berenbaum, 1987).

The toxic action of photodynamic compounds can be mediated by light activation of oxygen, notably singlet oxygen (1O_2), which can react at high rates with certain lipid, nucleic acid, and protein components of cells to cause disruption (Foote, 1976). HYP isolated from *Hypericum hirsutum* has been found to produce singlet oxygen (Knox and Dodge, 1985b).

Certain insects are capable of utilizing plants containing phototoxic compounds. Larvae of *Polia assimilis* (Lepidoptera: Noctuidae) feed on *Hypericum*, avoiding the phototoxic effects of HYP by adaptive behavior (Berenbaum, 1987), and it has been stated that *Chrysolina brunsvicensis* (Coleoptera: Chrysomelidae) may actually utilize HYP as a feeding cue (Rees, 1969).

The potential of synthetic photodynamic compounds for insect pest control has been reviewed (Heitz, 1982; Robinson, 1983; Lemke et al., 1987). However, the possibility of using natural photodynamic compounds for pest control programs has not been considered. Thus, insecticidal activity of HYP isolated from *Hypericum perforatum* towards a model pest species, larvae of the tobacco hawk moth *Manduca sexta* (Lepidoptera: Sphingidae), was investigated. Preliminary results from this report were presented at the ACS symposium "Light-activated pesticides" (Knox et al., 1987).

METHODS AND MATERIALS

Isolation of Hypericin. The HYP utilized in these experiments was isolated from *Hypericum perforatum*, which has HYP-containing glands on its leaves and stem. *H. perforatum* was grown from seed. The dried shoots were ground to a powder and extracted exhaustively in light petroleum ether with 5% (v/v) acetone to remove chlorophylls and carotenoids. The residue was allowed to dry, and the hypericin was extracted with 90% (v/v) aqueous acetone. The acetone fraction was washed repeatedly with light petroleum ether to remove the remaining photosynthetic pigments. The aqueous phase was acidified by addition of HCl. After 48 hr the dark precipitate was collected and washed with water. When dissolved in ethanol, the precipitate displayed the characteristic

absorption spectrum of HYP. *H. perforatum* contains pseudohypericin, which is indistinguishable from hypericin by means of absorption spectroscopy. For purposes of this study, no attempt was made to separate pseudohypericin from hypericin; the two compounds have equal $^1\text{O}_4$ -generation potential (data not shown). All concentrations of HYP were based on the compound's extinction coefficient in ethanol, $E_{592} = 41,600$.

Treatment of Manduca sexta Larvae. Larvae were reared at 25°C, 16 hr light–8 hr dark on artificial diet, using standard procedures (Bell and Joachim, 1978). Third-instar larvae that were actively feeding and weighed ca. 60 mg were used in all experiments, except the weight gain investigation, in which initial larval weight was ca. 150 mg, and larval development was monitored until the fourth instar.

HYP, in 5 μl of acetone, was applied to leaf disks of tobacco (7 mm diameter). The acetone was allowed to evaporate before feeding the disks to the larvae, which were maintained without illumination while consuming the disks (ca. 1 hr). Where appropriate, larvae were then fed artificial diet and exposed to light supplied by white fluorescent tubes (8 \times 40-W Thorn Warmlite tubes). Light levels were measured using a Li-Cor Radiometer with a pyranometer sensor. Irradiance was modulated by neutral (Kodak neutral density) and cutoff (Rank cinemoid) filters.

At least 15 larvae were used per dose of HYP. Larvae were weighed before treatment and, for certain experiments, at intervals following irradiation. Larvae were classified as dead by a lack of response to tactile stimulation. The LD_{50} was calculated by probit analysis.

RESULTS

HYP was phototoxic to *M. sexta* larvae. At the moderate radiance level used, the LD_{50} was 16 $\mu\text{g/g}$ larval initial fresh weight (fd. lmts. 14.37–18.05), which represents approximately a 1 μg dose to a third-instar larva. This dose was equivalent to HYP contained within four sepals of *H. hirsutum* (Knox and Dodge, 1985b). *H. perforatum* has an even higher shoot content of HYP.

It was noted that HYP-poisoned and then irradiated larvae stopped feeding and remained immobile until subjected to tactile stimulation, which induced irritability. The end result of hypericin poisoning is shown in Figure 1; growth of light-exposed larvae was retarded, and a characteristic discoloration, possibly due to oxidation of the tissues, was noted. The HYP-fed but dark-maintained controls and HYP-free irradiated controls attained normal body weight. These larvae continued development and pupated successfully.

The effect of HYP on weight gain was further investigated (Figure 2). Third-instar larvae were fed three low doses of HYP and then exposed to con-



FIG. 1. *Manduca sexta* larvae following hypericin poisoning. (A) Hypericin-fed dark-maintained control larvae. Hypericin-fed but irradiated larvae show characteristic discolorations (scale: mm).

stant light (22 W/m^2). Body weight was monitored over the following 76 hr. During the active feeding stage of the third instar the difference in body weight between $0.5 \mu\text{g}$ HYP-fed dark controls and $0.5 \mu\text{g}$ HYP-fed irradiated larvae was only 0.05 g. However, at 76 hr there was a significant difference in body weight (t test; $P < 0.01$). The lowest dose of HYP used ($0.12 \mu\text{g}$) also disrupted the normal growth pattern. There was no difference in body weight between HYP-fed dark-maintained controls and HYP-free irradiated controls (HYP-free data omitted in Figure 1 for clarity).

Reduced levels of irradiance resulted in reduced mortality (Table 1), and modulation of light quality by a cutoff filter (allowing irradiation with wavelengths $> 500 \text{ nm}$ only) reduced the mortality rate by only 20%, indicating that absorbance by HYP in the area of 500–600 nm was the major active region of the HYP action spectrum (for spectrum, see Knox and Dodge, 1985b). The fact that a 20% reduction did occur suggests that absorption of wavelengths $< 500 \text{ nm}$ could also elicit phototoxicity.

The phototoxic effect of HYP diminished rapidly when treated larvae were maintained in darkness for periods prior to irradiation (Figure 3). Mortality was reduced to 6% if irradiation was delayed for 8 hr after treatment. However, if

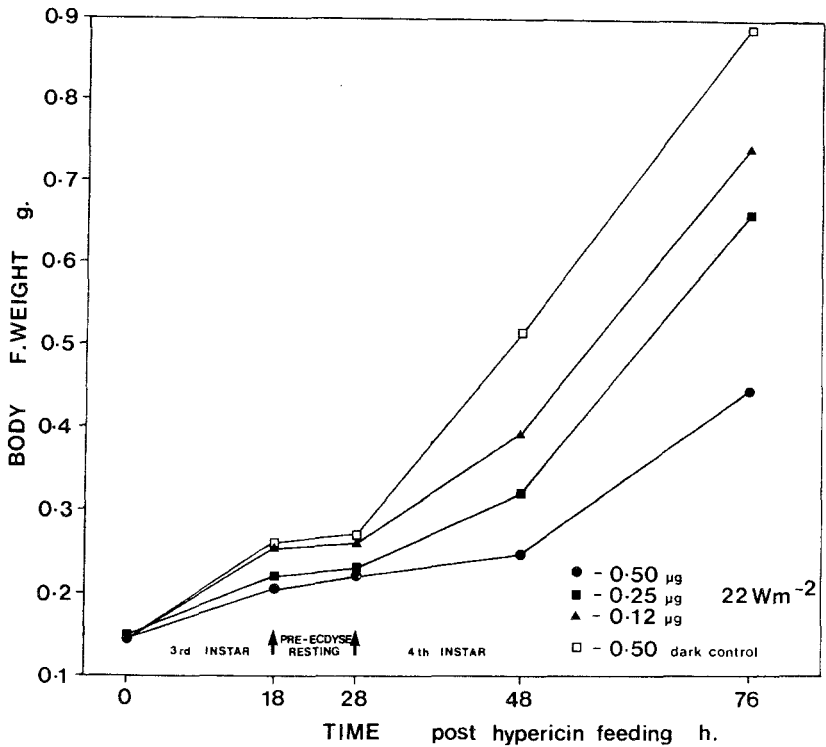


FIG. 2. The effect of sublethal doses of hypericin on larval weight gain. Controls were hypericin-fed ($0.5 \mu\text{g}$) and maintained in darkness and hypericin-free irradiated (not shown). Each data point was the mean of at least 15 individual larval body weight determinations.

TABLE 1. Phototoxicity of Hypericin toward *Manduca sexta* Larvae

Hypericin dose ($\mu\text{g}/\text{larvae}$) ^a	Irradiation (W/m^2)	Time in darkness between treatment and irradiation (hr)	Mortality at 48 hr (%) ^b
2.5	0	0	0
2.5	4	0	0
2.5	10	0	15
2.5	22	0	100
0	22	0	0
2.5	22	1 ^c	62
2.5	22	3 ^c	58
2.5	22	4 ^c	72

^a Mean initial fresh weight third-instar larvae approximately 60 mg.

^b Minimum of 15 larvae per data point. Data representative of two replicate experiments.

^c Larvae starved after hypericin treatment then retained in dark for stated time periods (hr) prior to irradiation and subsequent feeding on artificial diet.

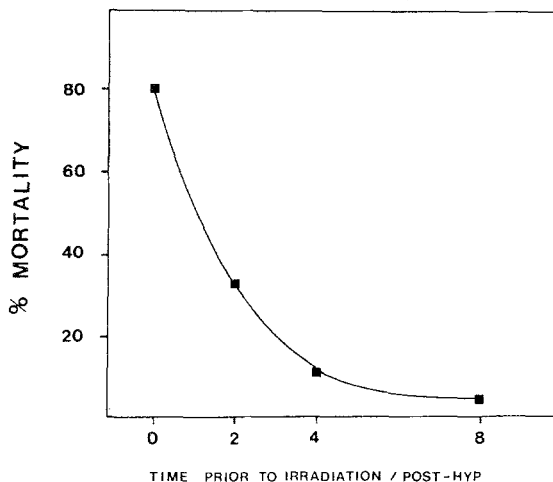


FIG. 3. Reduction in rate of mortality when larvae were retained without illumination for periods following hypericin poisoning. Larvae irradiated at 0, 2, 4, and 8 hr (15 insects in each group). Mortality recorded 48 hr after irradiation. No mortality seen for controls (HYP-fed and HYP-free).

larvae were not supplied with artificial diet following HYP treatment and retained without irradiation for short periods, the potential for a high mortality rate upon subsequent irradiation was retained (Table 1).

DISCUSSION

These studies demonstrate that orally administered HYP was highly toxic to *Manduca* larvae, an oligophagous herbivore unaccustomed to feeding on plants containing this compound. Photodynamic insecticidal activity of HYP has been noted previously only for mosquito larvae *Aedes atropalpus* (Arnason et al., 1983) and larvae of *Platynota flavedana* (Lepidoptera: Tortricidae) (Berenbaum, 1987).

Toxicity of HYP was entirely due to photodynamic action as there was a dependence upon light at the dose levels used in this study. Other photosensitizers may additionally act as light-independent toxins. Rose bengal adversely affects body weight gain in the boll weevil *Anthonomus grandis* without irradiation (Callahan et al., 1977). No effects on larval development were detected in hypericin-fed dark-maintained insects.

Growth retardation, an effect of HYP documented here, is one of the more common symptoms associated with photosensitized insects (Weaver, 1987). Ingestion of alpha-terthienyl was also found to affect *Manduca* larval development (Downum et al., 1984). However, alpha-terthienyl may possess antifee-

dant properties (Champagne et al., 1986), although this has yet to be fully established. Observations indicated that *Manduca* larvae were not deterred from feeding on HYP-poisoned tobacco leaf disks.

Hypericin-poisoned larvae remained completely immobile prior to death. However, if subjected to tactile stimulation, they became highly irritable. These behavioral abnormalities were indicative of disruption to the nervous system, one of the possible target sites of light-activated pesticides (Callaham et al., 1975).

Exposure to visible irradiation (500–600 nm) was a prerequisite for HYP toxicity, contrasting with other plant metabolites capable of photosensitizing lepidopteran larvae, such as alpha-terthienyl, which was toxic to *Manduca* orally and topically by subsequent exposure to UV-A irradiation (320–400 nm) (Downum et al., 1984). It is of interest to note that the maximum radiance levels used here for LD₅₀ studies (22 W/m²) were considerably less than normal daylight levels (> 200 W/m²), indicating a much lower effective dose requirement for insecticidal activity in a natural environment.

The rapid loss of toxic activity found when HYP-dosed larvae were allowed to feed in darkness prior to irradiation indicated either rapid breakdown or excretion. If fecal pellet production was prevented (larvae starved) so that ingested hypericin remained within the insect's body, the light-dependent toxicity was essentially retained. The slight reduction in toxicity observed was probably due to detoxification.

Secondary plant compounds may act as insecticides by deterring an insect from feeding, poisoning per se (independent of irradiation), or by the production of toxic molecules such as ¹O₂ in the presence of light. Hypericin is capable of the photodynamic generation of ¹O₂ in vitro (Knox and Dodge, 1985b). However, the role of ¹O₂ in its phototoxicity towards *M. sexta* is uncertain, and other radical mechanisms, possibly involving superoxide anions, may be important.

The precise reactions of hypericin and the sensitive sites of its phototoxic action at the tissue, cellular, and biochemical level have yet to be determined. Hypericin acts as a true phototoxin by producing highly damaging singlet oxygen in the presence of visible irradiation. Compounds such as hypericin provide an insight to the toxic mode of action of photodynamic compounds and also have potential as a new class of insecticides.

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COMPARATIVE ANALYSIS OF SEX-PHEROMONE-RESPONSE ANTAGONISTS IN THREE RACES OF EUROPEAN CORN BORER

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Abstract—Behavioral responses of males from three New York races of European corn borer, *Ostrinia nubilalis* (Hübner), to various suspected sex-pheromone-response antagonists were investigated with a flight tunnel protocol. Males from a bivoltine (Z) race and from a univoltine (Z) race utilizing a natural pheromone blend of 98:2 (Z)-:(E)-11-tetradecenyl acetates and males from a bivoltine (E) race utilizing a 1:99 (Z)-:(E)-11-tetradecenyl acetate ratio were flown to their natural blends and to blends containing 1% of one of the following additional compounds: Z9-12:OAc, E9-12:OAc, Z9-14:OAc, and E9-14:OAc. In each race, the added components lowered significantly the number of individuals completing the behavioral sequence. The only exception was bivoltine E males flown to a blend with E9-12:OAc added. The number completing the sequence in this case was statistically not significantly lower than the number that completed the sequence to the standard blend. In all three races, the Z9-14:OAc produced the most dramatic reduction in completed flights, and in the univoltine Z race, this added component was significantly more effective in reducing completed flights than any other added component. The response to the Z9-14:OAc is understandable given recently published findings of an electrophysiological study of single sensilla in the European corn borer.

Key Words—European corn borer, *Ostrinia nubilalis*, Pyralidae, Lepidoptera, (Z)-9-tetradecenyl acetate, pheromone, flight tunnel.

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INTRODUCTION

Three European corn borer (ECB), *Ostrinia nubilalis* (Hübner), races have been defined in New York (Roelofs et al., 1985). These include bivoltine and univoltine populations whose sex pheromone is a 98:2 mix of (Z)- and (E)-11-tetradecenyl acetates (Z11-14:OAc and E11-14:OAc) (Z races) (Klun et al., 1973; Glover et al., 1987), and a bivoltine population that uses a 1:99 blend (E race) (Kochansky et al., 1975; Glover et al., 1987). Although the Z races use a similar pheromone blend, males from these two races can be differentiated in the flight tunnel by their response to mixtures containing only 0.5% of the E isomer (Glover et al., 1987). Bivoltine males exhibited good flight responses to the 0.5% E blend (80% completing their flights compared to 100% completing their flights to the natural 3% E blend), whereas univoltine males responded poorly to this off blend (17% completing their flights compared to 90% completing their flights to the 3% blend). Thus, all three ECB races exhibited different behavioral response profiles to the set of pheromone blends used in the flight tunnel.

Recently, Struble et al. (1987) reported that the presence of 0.1% of (Z)-9-dodecenyl acetate (Z9-12:OAc) or (Z)-9-tetradecenyl acetate (Z9-14:OAc) can greatly reduce the capture of male ECB by the natural 100:3 Z/E blend in three areas of Canada. Some reduction also was observed with (E)-9-tetradecenyl acetate (E9-14:OAc), which previously had been shown to be effective at ca. the 3% level in reducing trap catch of Z-responding males in Iowa and cage bioassay responses of Iowa (Z) and New York (E) males (Klun et al., 1979). A number of other closely related isomers and homologs did not have any significant effect on the capture of male ECB. Since the trapping studies could not reveal the behavioral response effects of the added compounds, Struble et al. (1987) suggested that behavioral studies should be conducted with these compounds. Therefore, the present flight tunnel study was carried out to determine the behavioral effects of adding low amounts of Z9-12:OAc, Z9-14:OAc, E9-12:OAc, or E9-14:OAc to the natural blend of the three ECB races presently cultured in our laboratories and to determine if differential effects of these compounds on the male's response would allow us to discriminate further among the three ECB races.

METHODS AND MATERIALS

Insect Cultures. The univoltine Z culture was started from field-collected larvae, pupae, and adults from corn stubble near Paris, New York, during July 1984. The bivoltine Z culture was started from field-collected larvae, pupae, and adults from corn stubble near Eden, New York, during August 1984. The

bivoltine *E* culture was started from adults emerging from corn stubble collected near Geneva, New York, during May 1985. These three cultures have been reared continuously using the techniques and diet described previously (Roelofs et al., 1985), which were modified from Reed et al. (1972). Male pupae were isolated individually in 1.25-oz plastic creamers with a 2-cm piece of dental wick soaked with tap water. They were kept in continuous light at $30 \pm 1^\circ\text{C}$ until eclosion. After eclosion, all insects were entrained to a 16:8 light-dark photoperiod at $25 \pm 1^\circ\text{C}$ for two or three days in the absence of females before being used in the bioassays, with all insects acclimated to the flight tunnel room for 4–6 hr before being tested.

Pheromone Blend Sources. The *E*11–14:OAc was TLC-purified material and the *Z*11–14:OAc was purchased from Dr. S. Voerman (The Institute for Pesticide Research, The Netherlands). Both the *E*11–14:OAc and *Z*11–14:OAc were >98.6% pure by GLC analysis and both contain <0.05% of the opposite isomer. The *E*9–12:OAc, *Z*9–12:OAc, *E*9–14:OAc, and *Z*9–14:OAc were purchased from Farchan Division of ChemSampCo, Inc., Willoughby, Ohio. The standard *Z* blend consisted of 97% *Z*11–14:OAc and 3% *E*11–14:OAc, and the standard *E* blend consisted of 1% *Z*11–14:OAc and 99% *E*11–14:OAc. These blends were made in concentrations of 1 mg/ml using redistilled Skellysolve B (Skelly B) as solvent. The additional components (*E*9–12:OAc, *Z*9–12:OAc, *E*9–14:OAc, and *Z*9–14:OAc) were made up in 100 ng/ μl solutions using redistilled Skelly B as solvent. Ten microliters of each of these solutions was added to different solutions containing 100 $\mu\text{g}/100 \mu\text{l}$ of either the *Z* or *E* standard solutions. Capillary GLC analysis (30-m Supelcowax 10 column) verified that these blends contained 0.96–1.20% of the additional component. Thirty microliters of the standard blend or the standard blend plus an additional component was applied to 5×9 -mm rubber stoppers (Thomas Scientific, Swedesboro, NJ, catalog No. 8753-D22) so that the final amount was ca. 30 μg . Each stopper was allowed to stand in a hood 2–4 hr before its initial use in the flight tunnel. Thereafter each stopper was stored in a screw top glass vial at -20°C . Before each use a stopper was warmed to room temperature in its vial prior to introduction into the flight tunnel.

Flight Tunnel. The flight tunnel has been described previously by Glover et al. (1987). The wind speed during all flights was 0.25 m/sec and the illumination was 11 lux of red light at the tunnel floor. The distance from the pheromone source to the insect release site was 1.2 m. All flights were conducted at $18 \pm 1^\circ\text{C}$ and 3–5 hr into scotophase.

Flight Tunnel Protocol. Individual naive male insects were placed in 3-cm \times 6-cm screen cylinders, which in turn were placed on a 10-cm-high release platform with the open end of the cylinder upwind. Each insect was allowed 30 sec to initiate flight. Insects that did not activate within that time period but later demonstrated the ability to fly were recorded as not responding (NR). For

each responding insect, the final behavioral response was recorded using the following sequence of criteria: ACT = activation, rapid wing beating, and walking within the screen release cylinder; TF = taking flight; CAST = casting or looping flight near the release stand; OR = orientation flight in the pheromone plume; MID = flight in the plume to the midpoint of the tunnel; 30 cm = flight in the plume to within 30 cm of the pheromone source; 5 cm = flight in the plume to within 5 cm of the pheromone source; TS = touching the source, landing on the pheromone source stand, and/or touching the rubber stopper; DIS = display, clasper extrusion with wings held vertically, and abdomen waving slowly from side to side. Any insect touching the source or displaying was considered to have completed the behavioral sequence, so these two categories have been combined for the analyses.

For each culture, at least 41 insects were flown to each blend containing an additional component and at least 61 insects were flown to the standard blend. Data were collected on a minimum of four different days for each blend tested. The standard blends were utilized at the beginning of each flight day to check the tunnel conditions. Only on days when at least 70% of the insects completed the behavioral sequence to the standard blends were the additional component blends tested. Not all additional component blends were tested every day, and the order of the tests was randomized each day. The data summaries presented in this article include final behavioral responses for only those days on which both standard and additional component blends were tested.

Statistical Analysis. For each culture, the distribution of final behavioral responses to each blend containing an additional component was compared to the distribution of final behavioral responses to the standard blend for that culture using a one-tailed Kolmogorov-Smirnov two-sample test for large samples, n_1 and n_2 both > 40 (Siegel, 1956). This two-sample test is concerned with the agreement between two cumulative distributions and is sensitive to any kind of difference in the distributions. The test of significance for this test approximates a chi-square (χ^2) distribution with two degrees of freedom. In addition, within each culture, the percentages completing the behavioral responses, i.e., touching the source and/or displaying, to each of the five blends were compared using the simultaneous $\sqrt{\chi^2}$ procedure to generate confidence intervals for the simple contrasts. The general formula for these confidence intervals is

$$(\hat{p}_i - \hat{p}_j) \pm \sqrt{\chi_{k-1; 1-\alpha}^2} \sqrt{\frac{\hat{p}_i \hat{p}_i}{n_i} + \frac{\hat{p}_j \hat{p}_j}{n_j}}$$

where \hat{p}_i and \hat{p}_j are the proportions of samples i and j , respectively, completing the behavioral sequences. This calculation allows the overall probability of a type 1 error to be fixed for the entire set of confidence intervals. This post hoc

multiple comparison procedure for proportions is outlined more fully in Marascuilo and McSweeney (1977).

RESULTS

Univoltine Z. The univoltine Z males responded extremely well to the standard blend of 97% Z11-14:OAc and 3% E11-14:OAc, with 58 of 61 (95.1%) of the insects completing the behavioral sequence (Table 1, Figure 1A). In contrast, blends containing 1% of any of the additional components elicited very different response profiles (Table 1, Figure 1A). In all four cases, the profile (Figure 1A) was significantly different ($P < 0.005$) from the profile to the standard blend according to Kolmogorov-Smirnov two-sample tests (E9-14:OAc, $\chi^2 = 18.14$; Z9-14:OAc, $\chi^2 = 81.13$; E9-12:OAc, $\chi^2 = 30.54$; and Z9-12:OAc, $\chi^2 = 21.35$). Although the behavioral response profiles to

TABLE 1. FINAL BEHAVIORAL RESPONSE OF 2- to 3-DAY-OLD UNIVOLTINE Z STRAIN ADULT MALE *Ostrinia nubilalis* TO STANDARD PHEROMONE BLEND AND BLENDS CONTAINING 1% ADDITIONAL COMPONENTS

	Standard ^a	Standard plus			
		E9-14:OAc	Z9-14:OAc	E9-12:OAc	Z9-12:OAc
NR	0	2	6	1	0
ACT	0	0	0	0	0
TF	2	1	10	6	3
CAST	0	0	4	2	3
OR	0	0	5	2	2
MP	0	0	3	0	2
30 cm	0	1	2	0	3
5 cm	1	16	10	12	9
TS	14	5	1	12	11
DIS	44	17	1	7	12
N	61	42	42	42	45
Percent of sample touching source and/or displaying ^b	95.1 a	52.4 b	4.8 c	45.2 b	51.1 b

^aThe standard Z blend consisted of 97% Z11-14:OAc and 3% E11-14:OAc. Blends with additional components contained 99% standard blend and 1% additional material. All septa had a total of 30 μ g of material.

^bPercentages followed by the same letter are not significantly different at the 1% level using the post hoc multiple comparisons procedure of simultaneous $\sqrt{\chi^2}$ to generate confidence intervals for simple contrasts.

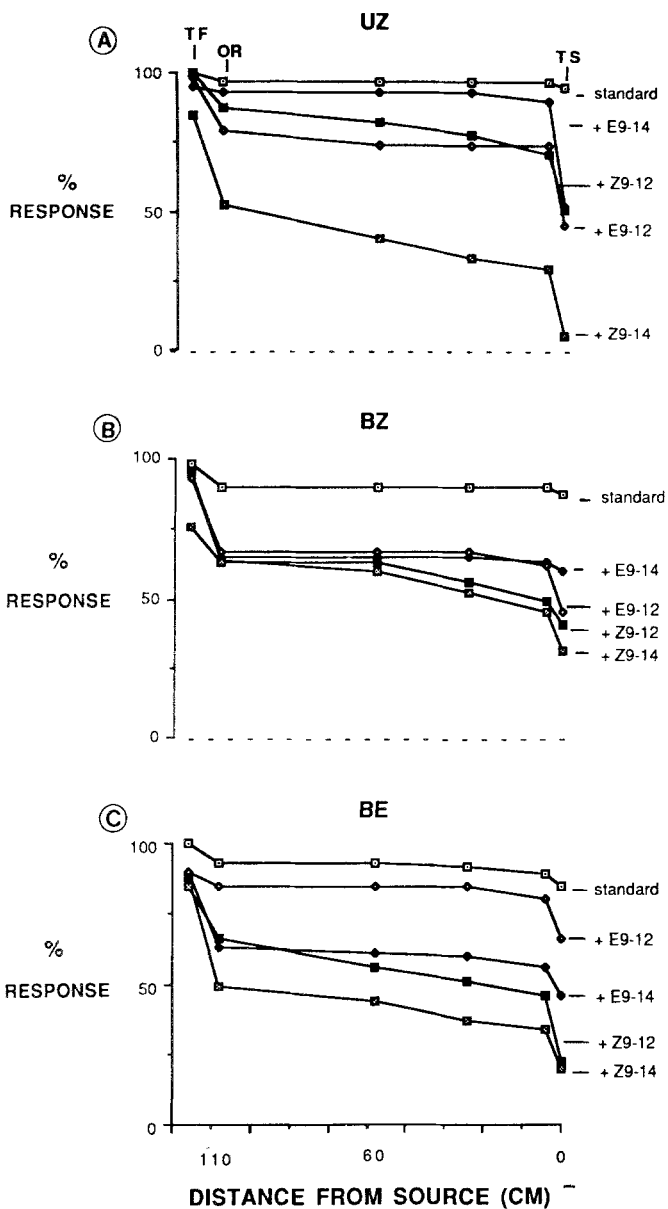


FIG. 1. Percentage response of male ECB from three races tested individually in the flight tunnel to 30- μ g dosages of standard pheromone blends and blends with 1% of an additional component. Behaviors are: taking flight (TF), orientation flight (OR), upwind flight to 60, 30, and 5 cm from the source, and touching the source (TS). Within each race, the response profiles to additional component blends were compared to the response profile to the standard blend via a Kolmogorov-Smirnov two-sample test. All response profiles to additional component blends were significantly different from the response profiles to the standards ($P < 0.005$) except the +E9-12:OAC in the BE race ($P > 0.1$).

the blends with 1% *E9-14:OAc*, 1% *E9-12:OAc*, and 1% *Z9-12:OAc* were similar (Figure 1A), they deviated dramatically from that of the standard blend by showing considerable arrestment at close range (5 cm). The insects flown to the blend with 1% *Z9-14:OAc* showed this same arrestment, but also had a significant proportion (48%) that failed to lock on the signal and therefore terminated flight before orienting to the blend plume (Figure 1A).

The percentage of insects completing the behavioral sequence, i.e., touching the source and/or displaying (Table 1), was significantly lower ($P < 0.01$) with any additional component than the 95.1% to the standard blend according to the post hoc multiple comparison procedure described earlier. The addition of *Z9-14:OAc* reduced the number of insects completing the behavioral sequence to 4.8%, whereas the addition of other components reduced the number completing this sequence to between 45.2 and 52.4%. This reduction with *Z9-14:OAc* compared with the other additional components is significant ($P < 0.01$).

Bivoltine Z. Fifty-four of 62 (87.1%) bivoltine males completed the behavioral sequence to the standard blend of 97% *Z11-14:OAc* and 3% *E11-14:OAc* (Table 2, Figure 1B). Again, blends containing 1% of any additional component elicited response profiles (Figure 1B) that were very different ($P < 0.005$) from the standard blend response profile according to Kolmogorov-Smirnov two-sample tests (*E9-14:OAc*, $\chi^2 = 12.30$; *Z9-14:OAc*, $\chi^2 = 35.94$; *E9-12:OAc*, $\chi^2 = 30.52$; and *Z9-12:OAc*, $\chi^2 = 20.53$). With all additional component blends, approximately one third of the insects did not orient to the blend plume. For *E9-14:OAc*, *Z9-12:OAc*, and *E9-12:OAc*, the number of insects terminating the behavioral sequence this early is markedly higher than in the results reported in Table 1 and Figure 1A for the univoltine *Z* population. The blends containing *Z9-14:OAc* and *E9-12:OAc* again elicited some close-range arrestment, with ca. 21% of the moths terminating flights at 5–30 cm from the blend source. This arrestment was less dramatic with *Z9-12:OAc* added to the standard blend.

The percentage of the insects completing the behavioral sequence (Table 2) was significantly lower than 87.1% for all additional component blends ($P < 0.05$ for *E9-14:OAc*, $P < 0.01$ for all others; post hoc multiple comparison procedures). Although the blend with *Z9-14:OAc* again yielded the lowest number completing the behavioral sequence (31.0%), this number was not significantly ($P > 0.05$) lower than those for the other additional component blends.

Bivoltine E. The behavioral responses of the bivoltine *E* strain to various blends are summarized in Table 3 and Figure 1C. The standard blend of 99% *E11-14:OAc* and 1% *Z11-14:OAc* elicited a complete behavioral sequence from 52 of 61 (85.2%) males exposed to it. According to the Kolmogorov-Smirnov two-sample test, the behavioral response profile for the blend contain-

TABLE 2. FINAL BEHAVIORAL RESPONSE OF 2- to 3-DAY-OLD BIVOLTINE Z STRAIN ADULT MALE, *Ostrinia nubilalis* TO STANDARD PHEROMONE BLEND AND BLENDS CONTAINING 1% ADDITIONAL COMPONENTS

	Standard ^d	Standard plus			
		E9-14:OAc	Z9-14:OAc	E9-12:OAc	Z9-12:OAc
NR	1	2	2	1	6
ACT	0	1	0	2	4
TF	5	11	11	9	3
CAST	0	1	2	2	2
OR	0	0	2	0	0
MP	0	0	3	0	3
30 cm	0	1	3	2	3
5 cm	2	1	6	7	3
TS	5	7	5	9	2
DIS	49	19	8	10	15
N	62	43	42	42	41
Percent of sample touching source and/or displaying ^b	87.1 a	60.5 b	31.0 b	45.2 b	41.5 b

^aBlends are the same as in Table 1.

^bPercentages followed by the same letter are not significantly different at the 5% level using the post hoc multiple comparisons procedure of simultaneous $\sqrt{\chi^2}$ to generate confidence intervals for simple contrasts.

ing an addition of 1% E9-12:OAc is not significantly different ($\chi^2 = 3.65$, $P > 0.1$) from the response profile for the standard blend (Figure 1C). However, the profiles for blends with the addition of E9-14:OAc, Z9-14:OAc, and Z9-12:OAc (Figure 1C) were all significantly different ($P < 0.01$) from the standard blend profile according to the same statistical procedure (E9-14:OAc, $\chi^2 = 14.84$; Z9-14:OAc, $\chi^2 = 42.34$; and Z9-12:OAc, $\chi^2 = 39.17$). The profiles in Figure 1C again demonstrate both the failure of a portion of the insects to lock on to the blend signal with Z9-12:OAc, E9-14:OAc, and Z9-14:OAc and some close-range arrestment (5-30 cm), most dramatically with Z9-12:OAc.

Whereas all blends with additional components elicited fewer completions of the behavioral sequence than did the standard blend (Table 3), the 65.9% completing the sequence with the E9-12:OAc additional component was not significantly lower ($P > 0.05$). All others were significantly lower ($P < 0.01$) with the response to the blend containing Z9-14:OAc again being the most markedly reduced. All contrasts for completion of the behavioral sequence were by the post hoc multiple comparison test described earlier.

TABLE 3. FINAL BEHAVIORAL RESPONSE OF 2- to 3-DAY-OLD BIVOLTINE *E* STRAIN ADULT MALE, *Ostrinia nubilalis* TO STANDARD PHEROMONE BLEND AND BLENDS CONTAINING 1% ADDITIONAL COMPONENTS

	Standard ^a	Standard plus			
		E9-14:OAc	Z9-14:OAc	E9-12:OAc	Z9-12:OAc
NR	0	4	4	3	3
ACT	0	0	1	1	3
TF	0	7	8	1	4
CAST	4	4	8	1	4
OR	0	1	2	0	4
MP	1	1	3	0	2
30 cm	2	1	1	2	3
5 cm	2	4	6	6	9
TS	12	3	4	7	4
DIS	40	16	4	20	5
<i>N</i>	61	41	41	41	41
Percent of sample touching source and/or displaying ^b	85.2 a	46.3 bc	19.5 c	65.9 ab	22.0 c

^aThe standard *E* blend consisted of 99% E11-14:OAc and 1% Z11-14:OAc. Blends with additional components contained 99% standard blend and 1% additional material. All septa had a total of 30 µg of material.

^bPercentages followed by the same letter are not significantly different at the 1% level using the post hoc multiple comparisons procedure of simultaneous $\sqrt{\chi^2}$ to generate confidence intervals for simple contrasts.

DISCUSSION

Behavioral analyses of males from three New York ECB races flown to pheromone blends containing 1% of additional components showed that there are significant differences in the antagonistic effects of these components among the races. In every case, except with E9-12:OAc effects on bivoltine *E* males, the additional component significantly lowered the percentage of individuals completing the behavioral sequence as compared to the natural blend. Blends with Z9-14:OAc added were the most active in all races and Z9-14:OAc was significantly more active than the other three additional components in tests of the univoltine *Z* race. Although we investigated these antagonists at somewhat higher ratios than were generally used in field studies by Struble et al. (1987), our results further confirm the antagonistic nature of Z9-12:OAc, Z9-14:OAc, and E9-14:OAc when added to standard blends of ECB pheromone. Further, we have demonstrated antagonism with the addition of E9-12:OAc. We, therefore, agree with the warning issued by Struble et al. (1987) about the possible

effects of contamination by small amounts of these compounds in commercial batches of pheromone used for monitoring of ECB populations.

A response to Z9-14:OAc in both the Z and the E races is understandable in view of the finding (Hansson et al., 1987) of a specialized olfactory cell in all three races that responds only to Z9-14:OAc. They did not find any specialized olfactory cells for the other antagonists utilized in this study, but Struble et al. (1987) did report a positive EAG response to both Z9- and E9-14:OAc. Olfactory cells that are very sensitive to trace amounts of a specific compound and that, when activated, adversely affect the male moth's normal behavioral responses to the sex pheromone blend also have been found in other species. It has been suggested that this phenomenon allows male moths to discriminate between heterospecific blends that contain the same major component (Linn et al., 1988). For example, both the cabbage looper moth (CL), *Trichoplusia ni* (Hübner), and the soybean looper moth (SBL), *Pseudoplusia includens* (Walker), utilize Z7-12:OAc as a major component in their six- and five-component blends, respectively (Linn et al., 1984, 1987b). Grant et al. (1987) found that SBL males possess an antennal olfactory receptor neuron that is specifically sensitive to low dosages of Z5-12:OAc. This compound is a minor component of the CL blend and is not found in the SBL blend, but it adversely affects the upwind flight response of SBL males in the flight tunnel (Linn et al., 1988). Additionally, O'Connell et al. (1983) found a neuron on male CL antennae that responds specifically to Z7-12:OH. This alcohol is not part of the six-component blend for CL, but it does cause significant levels of arrested upwind flight in the flight tunnel when present in the CL blend in amounts as low as 0.5% of the major component (Linn et al., 1984). Relative to this case, it is known that a related noctuid, *Autographa californica*, utilizes the same major component as CL, but apparently also uses the alcohol as an additional pheromone component (Steck et al., 1979).

Thus, there is increasing evidence that specific pheromone blends act as a unit in mediating the complete sequence of behavioral responses to a calling female (Linn et al., 1987a) but that an individual component of that blend can be detected specifically by and have an adverse affect on the male moths of another species.

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LEAF-TYING BY TORTRICID LARVAE AS AN
ADAPTATION FOR FEEDING ON PHOTOTOXIC
Hypericum perforatum

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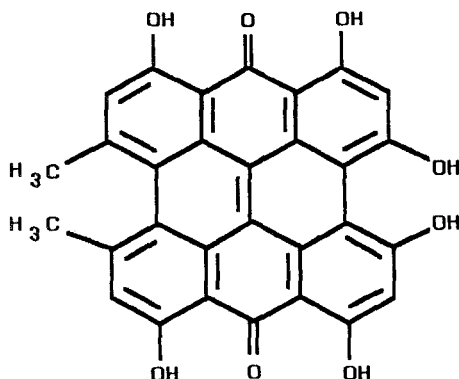
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Abstract—Hypericin, an anthrone dimer in *Hypericum perforatum* (Guttiferae), is shown for the first time to be phototoxic to generalist lepidopteran larvae; survivorship was reduced and development prolonged for *Heliothis zea* (Noctuidae) and *Platynota flavedana* (Tortricidae) that consumed hypericin diets in the presence of light. Survivorship for both species is enhanced when photoactivating wavelengths are excluded by filters. In nature *P. flavedana* successfully survives on *H. perforatum* by tying together leaves and feeding inside the ties. Shielded from light, the larvae are protected by their mode of feeding from phototoxic compounds.

Key Words—*Hypericum perforatum*, phototoxicity, hypericism, *Platynota flavedana*, Tortricidae, *Heliothis zea*, Noctuidae, Lepidoptera.

INTRODUCTION

The phototoxicity of St. John's-wort, *Hypericum perforatum* (Guttiferae), to sheep, cattle, and horses is attributable to the presence of the phototoxin hypericin (Scheme 1). Localized in glands of the plants' flowers, leaves, and stems (Blum, 1941; Giese, 1980), hypericin is an anthrone dimer and is classified as a primary photosensitizing agent (Clare, 1952). As such, hypericin is a highly active photodynamic pigment that reacts with light at the surface of the skin, causing hemolysis of red blood cells at concentrations as low as 10^{-7} M (Pace and MacKinney, 1941; Pace, 1942). Ingestion of the plant followed by exposure to sunlight results in the irritation of unpigmented skin, a condition known as hypericism, leading in severe cases to blistering, sloughing of necrotic areas,



HYPERICIN

SCHEME 1.

and, occasionally, death (Blum, 1941; Pace, 1942; Giese, 1980). Accordingly, hypericin appears to confer some protection for the plant since livestock generally avoid St. John's-wort, unless other forage is scarce (Harris and Peschkin, 1974). Little is known, however, as to whether hypericin also deters potential insect herbivores. To test this, bioassays were conducted with larvae of the corn earworm, *Heliothis zea* (Noctuidae), a polyphagous lepidopteran not known to feed on *H. perforatum* in nature.

A number of insects do utilize *H. perforatum* in its indigenous range, Eurasia (Currie and Garthside, 1932; Wilson, 1943; Johansson, 1962), but there are few records of native North American insects adopting *H. perforatum* as a host plant (Tietz, 1972; Hodges, 1974; Kingsolver et al., 1984). Thus, it was of interest to encounter larvae of several species of native North American tortricids (Lepidoptera) feeding within leaf ties on *H. perforatum* in central Illinois (Sandberg and Passoa, 1988). All tortricid species have broad host ranges and, for each, *H. perforatum* represents a new host record. Bioassays were conducted on one of these species, *Platynota flavedana*, to examine possible mechanisms that allow the development of a generalist feeder on a plant containing a phototoxin.

METHODS AND MATERIALS

Extraction and purification of hypericin from *Hypericum perforatum* were based on a modification of a procedure developed by Knox and Dodge (1985).

Flowers, upper leaves, and stems were immersed into a series of beakers containing 90% aqueous acetone, until the intense red color was no longer produced. The acetone extracts were combined and filtered through Whatman No. 1 filter paper to remove flower and leaf material. Chlorophyll and carotenoids were removed by partitioning the aqueous acetone solution with petroleum ether (bp 30–60°C). The aqueous solution was then brought to dryness by rotary evaporation followed by lyophilization. This formed the crude extract in one of the bioassays.

The hypericin extract was further purified using a gravity column 40 mm in diameter with a 10 cm layer of 32- to 63- μm silica on the bottom and a 1- to 2-cm layer of sand on top. The dried *Hypericum* extract was then resolubilized in 100% acetone, loaded on top of the layer of sand, and the column was run with ether-acetone in a 1:1 ratio. After the pink-red fractions were collected, they were combined, brought to dryness, and purified by thin-layer chromatography on a 2-mm, 20 \times 20-cm silica gel 60 F254 plate. The solvent systems used consecutively were ether-acetone at a ratio of 1.5 : 1.0 and ether-methanol at a ratio of 9 : 1 with R_f s of 0.88 and 0.17, respectively. The red band was scraped from the plate and eluted with ethanol. The solution was brought to dryness, and the resulting dry powder was used in artificial diet studies. Throughout the extraction and purification procedures, a spectrophotometer was used to monitor the presence of hypericin by using hypericin's characteristic absorption peaks between the region of 450–600 nm of the visible light spectrum (Schiebe and Schontag, 1942; Giese, 1980). The possibility exists that some pseudohypericin, a closely related pigment with an absorption spectrum identical to that of hypericin, may have been present in the hypericin preparation, although thin-layer chromatography did not indicate its presence. Nonetheless since pseudohypericin is also a photosensitizer, its presence would not adversely affect the bioassays or their interpretation (Giese, 1980; Knox and Dodge 1985).

In order to bioassay hypericin for insecticidal activity, a modified Vanderzant-wheat germ artificial diet (Waldbauer et al., 1984) was developed for *Heliothis zea* and *Platynota flavedana*, with wheat germ as the only undefined ingredient for the *H. zea* diet. The foliage of strawberry, one of the hosts of *Platynota*, is not reported to contain phototoxins (no phototoxic effects to survivorship were observed in the controls in subsequent experiments) and was added to the *Platynota* diet to stimulate larval feeding.

An initial bioassay of hypericin tested for insecticidal activity towards *Heliothis zea*, a generalist insect not known to feed on *Hypericum*. Larvae were reared to second instar on control diets and diets containing 0.1% fresh weight under two different light treatments. The first light treatment, fluorescent lights (Sylvania Cool White 40 watt) with a clear filter, simulated sunlight, and the second, fluorescent lights with a red acetate filter, filtered out wavelengths below

600 nm, the activating range of the light spectrum for hypericin (Schiebe and Schontag, 1942; Giese, 1980).

The second bioassay examined the response of *Platynota flavedana*, a generalist insect known to feed on *Hypericum*. The larvae were subjected to three artificial diet treatments: a control, one with *Hypericum perforatum* leaves (0.075% fresh weight), and one containing a crude extract from the equivalent weight of *Hypericum* leaves. The extract was prepared as described in the purification procedure of hypericin. As it was already known that *P. flavedana* is able to utilize *Hypericum*, the leaves and a crude extract containing hypericin were assayed to determine if either could be toxic to *Platynota* if larvae were constrained to feed on the diets under conditions simulating sunlight (i.e., outside of a leaf-tie).

In three subsequent experiments, purified hypericin from *H. perforatum* was bioassayed against *P. flavedana*. Larvae were exposed to the same light treatments and reared to the second instar on three different levels of hypericin (0.02, 0.04, and 0.20% hypericin/fresh weight diet). Levels of hypericin in the flowers and leaves of *H. perforatum* have been reported to be approximately 0.03% (Brockmann and Sanne, 1957), 0.04% (Pace and MacKinney, 1941), and 0.05% (Brockman et al., 1942) fresh weight. Larvae were reared from egg hatch to the second instar as the previous experiment had shown the first instar to be the most vulnerable stage.

The survivorship data were analyzed as nonparametric three-way contingency tables by the log likelihood statistic G^2 . In each of the experiments, an equal number of larvae were exposed either to the control diet or the hypericin diet under the conditions of either "sunlight" or filtered light. Thus, by necessity, the totals of one margin of the contingency analyses were fixed, presetting the two-way interaction between diet and light at zero. Of interest in these experiments, however, is how survivorship is affected by diet, light, and both parameters conjointly. In the contingency analyses, the interaction between diet and light [DL] is present in all models, leaving only four possible models (instead of the usual eight) to explain the observed data (Fienberg, 1977).

RESULTS

The first experiment monitored the survivorship of the generalist *H. zea* on control diets vs. diets containing 0.10% hypericin. Survivorship was 100% on control diet in both light treatments and on the hypericin diet in filtered light. On the hypericin diet under nonfiltered light simulating sunlight, however, survivorship dropped to 8%. The first model of the three-way contingency analysis, [S] [DL], tests for independence of survivorship from diet and light treatment. The extremely large G^2 of 129.11 indicates that there is a large

amount of variability in the data not explained by the first model (Table 1). Models 2 and 3 test for the interactions of survivorship with diet and survivorship with light, respectively. Neither of these two-way interactions by themselves suffice to explain the data, although subtraction of their G^2 values from that for the initial [S] [DL] model indicates that both [DS] and [LS] are highly significant two-way interactions. The final model (4) tests for the sufficiency of these two-way interactions as descriptors of the data, acceptance of this model implying that no three-way interaction need be inferred. In this instance, the remaining G^2 is conventionally significant, so that the existence of a three-way interaction is inferred. In other words, light affects the survivorship observed on each diet; i.e., high mortality is dependent on exposure of the larvae to both the hypericin diet and nonfiltered light simultaneously, whereas low mortality is observed on the hypericin diet and filtered light.

In the bioassay of the *Hypericum* leaves and extract on *Platynota flavedana* (Table 2), the large G^2 of 23.38 again indicates that there is a large amount of variability in the data not explained by the first model. The two-way interactions of survivorship and light and survivorship and diet obtained by subtraction in models 2 and 3 were again both significant. The low G^2 of 3.79 in model 4 indicates that the two two-way interactions by themselves suffice to describe the data and that a three-way interaction need not be inferred, although this inference (that light and diet act jointly to affect toxicity) hinges on a borderline ($p = 0.15$) probability.

In the final series of experiments, larval survivorship of *P. flavedana* was independent of light and diet at the lower two dosages of hypericin, although there was higher mortality of larvae on the 0.04% hypericin diet under nonfiltered light than under filtered light (Table 3). The highest dosage of 0.20%

TABLE 1. THREE-WAY CONTINGENCY TABLE ANALYSIS OF *Heliothis zea* LARVAE REARED TO SECOND INSTAR ON CONTROL AND 0.10% HYPERICIN (FRESH WEIGHT) DIETS UNDER TWO LIGHT CONDITIONS ($N = 40$ FOR EACH TREATMENT)

Model ^a	G^2	df	Probability ^b	Two-way interactions
1. [S] [DL]	129.11	3	0.000	
2. [DS] [DL]	77.40	2	0.000	[DS] = 129.11 - 77.40 = 31.71 $P < 0.0005$ with 1 df
3. [LS] [DL]	77.40	2	0.000	[LS] = 129.11 - 77.40 = 31.71 $P < 0.0005$ with 1 df
4. [DS] [LS] [DL]	4.75	1	0.029	

^aS = survivorship, D = diet, L = light.

^bSee text for interpretation of probability values.

TABLE 2. PERCENT SURVIVORSHIP AND THREE-WAY CONTINGENCY TABLE ANALYSIS OF *Platynota flavedana* LARVAE REARED TO PUPATION ON CONTROL, *Hypericum perforatum* LEAVES AND *H. perforatum* EXTRACT DIETS UNDER TWO LIGHT CONDITIONS

	Survivorship (%)			
	Control diet (N)	Hypericum leaf diet (N)	Hypericum extract diet (N)	
Clear filter	89.5 (19)	45.0 (20)	60.0 (20)	
Red filter	94.7 (19)	94.7 (19)	77.8 (18)	
Three-way contingency table analysis				
Model ^a	G ²	df	Probability ^b	Two-way interactions
1. [S] [DL]	23.38	5	0.000	
2. [DS] [DL]	14.56	3	0.002	[DS] = 23.38 - 14.56 = 8.82 P < 0.005 with 2 df
3. [LS] [DL]	12.99	4	0.011	[LS] = 23.38 - 12.99 = 10.39 P < 0.05 with 1 df
4. [DS] [LS] [DL]	3.79	2	0.151	

^aS = survivorship, D = diet, L = light.

^bSee text for interpretation of probability values.

TABLE 3. PERCENT SURVIVORSHIP OF *Platynota flavedana* LARVAE REARED TO SECOND INSTAR ON CONTROL, 0.02% (N = 50), 0.04% (N = 50), AND 0.20% (N = 40) HYPERICIN (FRESH WEIGHT) DIETS^a

	Survivorship (%)					
	Control diet	0.02% diet	Control diet	0.04% diet	Control diet	0.20% diet
Clear filter	90	94	88	80	85	50
Red filter	96	90	90	88	80	78
Three-way contingency table analysis						
Model ^b	G ²	df	Probability ^c	Two-way interactions		
1. [S] [DL]	14.29	3	0.003			
2. [DS] [DL]	7.02	2	0.030	[DS] = 14.29 - 7.02 = 7.27 P < 0.01 with 1 df		
3. [LS] [DL]	11.70	2	0.003	[LS] = 14.29 - 11.70 = 2.59 P < 0.20 with 1 df		
4. [DS] [LS] [DL]	4.30	1	0.038			

^aThree-way contingency table analysis for control vs. 0.20% hypericin diet.

^bS = survivorship, D = diet, L = light.

^cSee text for interpretation of probability values.

hypericin yielded similar results as the initial experiment with *H. zea*. A significant two-way interaction was found between survivorship and diet but not between survivorship and light. The G^2 of 4.30 in model 4 again indicates that the two-way interactions are not sufficient to describe the data and that a three-way interaction must be inferred; that is, high mortality results from exposure to hypericin and sunlight conjointly.

Mean development time (in days) of *P. flavedana* larvae to second instar was analyzed by two-way analysis of variance in each of the three experiments bioassaying the purified hypericin (Table 4). Light consistently prolonged larval development time. On the control diets, development time ranged from 0.7 to 1.2 days longer in simulated sunlight versus filtered light. Similar results were obtained on the lower two dosages of hypericin. On the 0.20% hypericin diet, however, larvae that survived required an additional four days to molt to second instar under "sunlight" as compared to larvae under filtered light. In addition, at this hypericin level, the factor of diet and the interaction of diet \times light also significantly contributed to longer larval development time. Under the clear filter, larval development to second instar was delayed by nearly five days on the hypericin diet compared to the control diet. Larvae on the hypericin diet under the red filter, however, were only two days behind the control diet and only one day longer than those on control diet in "sunlight."

TABLE 4. MEAN DEVELOPMENT TIME OF *Platynota flavedana* LARVAE TO SECOND INSTAR (DAYS)

Diet	Time (days)		Two-way analysis of variance				
	Clear Filter	Red Filter	Source	df	MS	F	P
1. Control	6.93	5.77	Diet	1	2.809	0.67	0.4125
Hypericin (.02%)	7.09	6.11	Light	1	52.714	12.66	0.0005 ^a
			D \times L	1	0.398	0.10	0.7574
			Error	180	4.163		
2. Control	8.20	7.51	Diet	1	3.192	0.58	0.4470
Hypericin (.04%)	8.72	7.52	Light	1	38.828	7.07	0.0086 ^a
			D \times L	1	2.923	0.53	0.4667
			Error	171	5.494		
3. Control	7.26	6.44	Diet	1	329.649	58.83	<0.0001 ^a
Hypericin (.20%)	12.05	8.52	Light	1	133.074	23.75	<0.0001 ^a
			D \times L	1	51.260	9.15	0.0031 ^a
			Error	113	5.603		

^aSignificant at $P < 0.05$.

DISCUSSION

The mortality observed for both *P. flavedana* and *H. zea* on hypericin diets exposed to visible light provides evidence that hypericin is phototoxic to insects. The results were particularly dramatic for *H. zea*, a polyphagous lepidopteran not known to feed on *Hypericum perforatum*. Yet despite the toxicity of hypericin to *P. flavedana*, this species commonly utilizes *H. perforatum* as a host plant (Sandberg and Passoa, 1988). In the presence of simulated sunlight on diets containing *Hypericum* leaves, extract, or 0.2% hypericin, larvae either died or experienced significantly longer development time. When reared under filtered light, however, in which the activating wavelengths of hypericin were excluded, there was no difference in survivorship compared to the controls, and only a slightly longer development time. These results suggest that leaf-tying in nature may serve the same function as do the red filters in the laboratory. Leaves are essentially opaque to visible wavelengths (Gates, 1980), and a leaf-tie would therefore protect the larvae from the activating wavelengths of hypericin. The longer development times observed in nonfiltered light on both control diets and hypericin diets by *P. flavedana* may further reflect the fact that these larvae normally feed sheltered from light.

These experiments provide the first experimental evidence that a behavioral preadaptation may allow an insect to exploit a phototoxic plant. Berenbaum (1978) first suggested leaf-rolling as a means of protection from phototoxins for oecophorid specialists on furanocoumarin-containing plants. Champagne et al. (1986) also hypothesized concealed feeding as a means of protection for the larvae of the European corn borer, *Ostrinia nubilalis*. A polyphagous lepidopteran species, *O. nubilalis* may protect itself from the phototoxic polyacetylenes of its host plants in the Compositae by boring into stems or spinning profuse amounts of silk. Avoidance of the phototoxic effects of hypericin by feeding within a shelter may not be an uncommon ploy of other lepidopteran larval feeders on *H. perforatum*. Three other leaf-tying polyphagous tortricids were collected in association with *P. flavedana* on *H. perforatum* in Illinois (Sandberg and Passoa, 1988). Moreover, of 44 lepidopteran species recorded on *Hypericum* (principally from its native range in Eurasia), 25 are concealed feeders, protected from light; of these, 15 are leaf-rollers or tiers, three are case-makers, two are leaf-miners, two are stem-borers, two are seed-borers, and one is a flower-tier (Table 5) (Currie and Garthside, 1932; Wilson, 1943; Johannson, 1962; Hodges, 1974; Bradley et al., 1973, 1979; Sandberg and Passoa, 1988). Since *H. perforatum* was introduced into North America in the grass and grain seeds of early colonists (first recorded in 1785 in Essex County, Massachusetts; Marsh and Clawson, 1930) native Lepidoptera (including the four species of tortricids currently found feeding on *H. perforatum*) have

TABLE 5. LEPIDOPTERAN ASSOCIATES OF *Hypericum* THAT HAVE CONCEALED LARVAL FEEDING HABITS

Species	Family	Mode of feeding	Reference ^a
<i>Nepticula septembrella</i>	Nepticulidae	leaf miner	1,2,3
<i>Adela violella</i>	Incurvariidae	seed borer	2,3
<i>Nematois dumeriliella</i>	Incurvariidae	case maker	2,3
<i>Cemiosstoma lustratella</i>	Tineidae	case maker	3
<i>Nematois cupriacella</i>	Tineidae	case maker	3
<i>Leucoptera lustratella</i>	Lyoniidae	leaf miner	2
<i>Gracillaria aurogutella</i>	Gracillariidae	leaf roller	1,2,3
<i>Agonopterix hypericella</i>	Oecophoridae	leaf tier	1,2,3
<i>Agonopterix hyperella</i>	Oecophoridae	leaf tier	4
<i>Agonopterix lythrella</i>	Oecophoridae	leaf tier	4
<i>Agonopterix nubiferella</i>	Oecophoridae	leaf tier	4
<i>Depressaria liturella</i>	Oecophoridae	leaf tier	3
<i>Aristotelia atrella</i>	Gelechiidae	stem borer	2,3
<i>Aristotelia morphochrama</i>	Gelechiidae	stem borer	2
<i>Archips rosana</i>	Tortricidae	leaf tier	2,5
<i>Cacoeciamorpha pronubana</i>	Tortricidae	leaf tier	2,5
<i>Choristoneura parallela</i>	Tortricidae	leaf tier	7
<i>Cnephasia conspersana</i>	Tortricidae	flower tier	2,5
<i>Eucosma cana</i>	Tortricidae	seed borer	3,6
<i>Lathronympha hypericana</i>	Tortricidae	leaf tier	1,2,3
<i>Platynota flavedana</i>	Tortricidae	leaf tier	7
<i>Sesmasia hypericana</i>	Tortricidae	leaf tier	8
<i>Sparganothis sulfureana</i>	Tortricidae	leaf tier	7
<i>Tortrix divulsana</i>	Tortricidae	leaf tier	3
<i>Xenotemna pallorana</i>	Tortricidae	leaf tier	7

^aReferences: 1. Johannson, 1962; 2. Wilson, 1943; 3. Currie and Garthside, 1932; 4. Hodges, 1974; 5. Bradley et al., 1973; 6. Bradley et al., 1979; 7. Sandberg and Passoa, 1988; 8. Nelson, 1962.

had only a brief period of association with *H. perforatum* and hence little opportunity to coevolve with the plant. Leaf-tying, leaf-rolling, leaf-mining, and stem-, root-, fruit-, and seed-boring, however, are all characteristic feeding modes of tortricid larvae (Roelofs and Brown, 1982). These larval feeding behaviors initially may have been responses to pressures inflicted by predators and parasitoids (Weed, 1930; Price, 1980; Powell, 1980) and/or mechanisms to optimize the microclimate of the developing larva (Chauvin, 1967). Irrespective of the evolutionary forces that may have selected for their internal feeding habits, some microlepidopteran larvae with broad host ranges may be preadapted for feeding on plants containing phototoxins.

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EXPERIMENTAL HABITAT SCENTING INHIBITS COLONIZATION BY BEAVER, *Castor canadensis*

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Abstract—Unoccupied beaver (*Castor canadensis*) sites in New York State were for two years experimentally scented with a mixture of beaver castoreum and anal gland secretion. These sites were colonized less often than unscented control sites. The beaver is the first mammal to have been shown experimentally to use intraspecific odor cues when settling in vacant habitat. Territorial pheromones may be useful as repellents for beaver or other rodents.

Key Words—Adirondacks, beaver, *Castor canadensis*, castoreum, nuisance beaver, odors, pheromone, repellent, scent marking, territory, wildlife damage.

INTRODUCTION

Beaver maintain family territories year round and in spring and summer mark them with small scent mounds at the water's edge. With their front paws, the beaver scoop up mud and sticks from the pond, place them on land, and then deposit a mixture of castoreum from the castor sacs and secretion of the anal glands (AGS) on the mound (Aleksiuk, 1968; Hodgdon, 1978; Butler and Butler, 1979; Bollinger et al., 1983; Muller-Schwarze and Heckman, 1980; Svendsen, 1980; Muller-Schwarze et al., 1983).

Beaver scent marks most likely serve several different functions, such as advertisement of territory, orientation within the territory, sex recognition, sex attraction, advertising colony size or composition, or marking food. Aleksiuk (1968) proposed a territorial function, specifically to decrease conspecific contact. Indeed, colonies with closer neighbors constructed more scent mounds than more isolated colonies (Butler and Butler, 1979; Muller-Schwarze and

Heckman, 1980). A scent mark may provide an intraspecific warning signal ("no trespassing") or even a "psychological fence," both decreasing the probability of an agonistic encounter with its risk of injury or even death. As proposed for other mammals (Ewer, 1968) and experimentally demonstrated for rabbits (Mykytowycz et al., 1976), Svendsen (1980) suggested that scent marks affect the motivational state of beaver: They strengthen confidence and reduce anxiety in territory owners, but decrease confidence and increase the readiness to flee in all other beaver. Since beaver place their scent mounds at strategic points within their territories, such as trailheads, feeding sites, or dams (Muller-Schwarze and Heckman, 1980), they may also use them as orientation beacons (Collins, 1976). Finally, beaver discriminate between male and female gland secretions (Hodgdon, 1978; Bollinger et al., 1983). This suggests that this species' scent marks carry individual and sexual information.

The castoreum of the castor sacs consists of numerous compounds, of which over 40 have been identified in the Eurasian beaver, *C. fiber*. These include alcohols, ketones, phenols, aldehydes, and amines, notably "castoramine" (Lederer, 1949). Some major constituents are assumed to be derived from the diet and may vary greatly in concentration, including total absence. The other component of the scent mark is the oily secretion from the anal glands (Døving, personal communication). Gronneberg (1979) found over 60 different wax esters in the AGS of the *C. fiber*. The biological roles of these compounds from either source remain unknown. Recent bioassay experiments showed that several chemical fractions of castoreum release scent marking in resident beaver if the samples are placed in their territories (Muller-Schwarze et al., 1986).

Current beaver population expansion in the American Northeast causes increasing damage to developed land by flooding and destruction of valuable trees. Beaver already had been declared a "major nuisance" in five southern states (Larson and Gunson, 1983) and have also been a problem in several northern states (Parsons, 1978; Peterson and Payne, 1986). Each spring, 2-year-old beaver leave their parental territories and try to establish new colonies, either nearby or by dispersing to sites as far as 320 km away (Libby, 1957). They may settle in any suitable, but increasingly more marginal, site and typically modify it by impounding water. Agricultural or forest lands are often flooded, and roads or railroads damaged by softening of the soil. "Nuisance beaver" traditionally have been removed by live-trapping and transplanting. This, however, has become more expensive and available sites for new introductions are hard to find. Hence a need arose to find new, and more economic means of managing beaver, especially by manipulating their behavior.

This study examines the responses of transient beaver to artificially scented vacant beaver sites. It extends an earlier pilot experiment in Acadia National Park in Maine (Muller-Schwarze and Heckman, 1980) to a managed beaver population of lower density. We asked the question whether beaver are deterred

by artificial scent mounds that mimic site occupancy by an adult pair of beaver. If so, such a territorial demarkation signal may be a potential repellent, in principle, also for other rodents.

METHODS AND MATERIALS

Study Area. The study area was 1287-km² Fulton County, located in the southern foothills of the Adirondack Mountains in New York State. The county-wide beaver population is maintained at 30% maximum occupancy through a controlled trapping program by the New York State Department of Environmental Conservation. Sixty-six percent of the active colonies were located between 0.5 and 2.325 km from their nearest neighbors, with a median density of 0.5 colonies per stream kilometer (Welsh, unpublished observations). Compared with the estimated saturation density of 1 colony/km in northeastern hardwood forests (Nordstrom, 1972), this population is well below saturation level.

Woody vegetation was varied and consisted mainly of northern hardwoods such as beech (*Fagus grandifolia*), maple (*Acer* spp.), and yellow birch (*Betula lutea*), with hemlock (*Tsuga canadensis*), and balsam fir (*Abies balsamea*), on poorer sites. Almost all beaver sites contained some willow (*Salix* spp.), and alder (*Alnus* spp.). Stream gradients were low ($0.90 \pm 0.16\%$) and considered optimal for beaver. The county in general consists of numerous lakes, forests, developed towns and associated rural communities.

Experimental Procedure. The experiment took place in 1983 and 1984. The actual scenting occurred between May 2 and August 24, 1983, and between April 28 and July 31, 1984, the time of the year when transient beaver are most prevalent. Potential study sites were selected during the winters before the two field seasons. Sites were rated according to their resources, including woody vegetation as potential food, water supply, presence and condition of old beaver lodges and dams, and proximity to active beaver sites. Potential sites that were most similar in these categories were paired for two treatments: one site was scented, the other left as control. To keep the sample size large enough for statistical evaluation, we decided not to add a control odor as a third treatment. Care was taken that scented and control sites alternated on any given stream system, so that transient beaver always had a choice between two roughly equivalent sites that differed only in their treatment. Such a pair of sites could be located up- and downstream of an active colony, or on both arms of a stream fork. Fifty sites were chosen for study (24 experimental and 26 control) in 1983, and 46 sites (23 experimental, 23 control) in 1984.

The scent used in the experiment consisted of homogenized castor sacs and anal glands from adult beaver trapped the previous winter from areas in New York State that were well outside the study area. Ten pairs of glands, from five

males and five females, were blended to mimic a "mated pair odor." The resultant paste was transferred to Teflon bottles and stored at -10°C .

At the onset of the field season, six artificial scent mounds were constructed at each site along the water's edge by transferring stream substrate with a 1-liter plastic tub onto the shoreline. Experimental mounds then received 1 g of the castoreum-AGS mixture; the control mounds remained unscented. Two mounds each were placed beside the old lodge if one existed, on the shoreline opposite the lodge, and on either side of the main dam (or remnants of a dam), as well as 20 m both upstream and downstream of the center of the site, covering the potential immigration routes.

Every 14 days, each site was examined for signs of beaver occupancy such as fresh cutting, dam or lodge building, feeding, tracks, feces, or scent mounds. All mounds were rebuilt and sites rescented. When water levels dropped, old mounds were destroyed and new ones placed near the new waterline. We compared the results by a 2×2 test for independence with the G statistic.

RESULTS AND DISCUSSION

During the two years of the study, more control sites than scented sites were colonized ($P < 0.05$; G test; Table 1). This result is striking if one considers the many potentially intervening variables, notably a severe drought in summer 1983 and localized heavy fur-trapping. The drought affected the study by completely drying up 20% of the sites. In another 16% the water dropped to a level that probably hampered a beaver's foraging in the surrounding woodland.

Since only 30% of the county's suitable beaver sites were occupied, the beaver had a wide choice of alternative sites and could afford to avoid many of

TABLE 1. NUMBERS OF SCENTED AND CONTROL SITES COLONIZED BY BEAVER, *Castor canadensis*, DURING EXPERIMENT IN ADIRONDACK FOOTHILLS, FULTON COUNTY, NEW YORK^a

Year	Scented sites				Control sites		
	Colonized (%)	Remained vacant	<i>N</i>	Colonized (%)	Remained vacant	<i>N</i>	
1983	0 (0)	24	24	4 (15)	22	26	
1984	2 (9)	21	23	4 (17)	19	23	
Total	2 (4)	45	47	8 (16)	41	49	

^aDifference between scented and control sites: $G = 3.998$ ($df = 1$, $P < 0.05$).

our study sites. If, on the other hand, the population density were very high, even less attractive sites would likely be colonized, including those with beaver scent. In fact, an unexploited beaver population in a similar Eastern hardwood ecosystem in Allegany State Park, Cattaraugus County, New York, reached a density of 1.03 colonies/km of stream (Muller-Schwarze et al., 1986). There beaver colonized marginal habitat with stream gradients of up to 6.44%. In this case an attempt to prevent colonization of four sites by artificial scenting failed (Muller-Schwarze, unpublished observations). Thus, if competition for space is intense, scenting will not prevent transients from settling. However, if colonizing beaver have a choice, we believe a conspecific odor can be effective as a repellent.

The results of the present study together with its precursor (Muller-Schwarze and Heckman, 1980) demonstrate for the first time that experimentally used conspecific odor cues, the putative "territorial marks," will affect colonization by a free-ranging wild mammal. In birds, an experiment with auditory cues has shown that conspecific song from loudspeakers can delay the moving of male great tits (*Parus major*) into empty territories (Krebs, 1976).

Our study suggests that pheromones can deter beaver and possibly other rodents or larger mammals from entering areas where they may become a nuisance. To consider pheromones for practical applications, several biological conditions must be met: First, the animals should not habituate to the odor during the period of application, usually a complete breeding season. The avoidance response is most likely stronger if reinforced by encounters, particularly hostile, with conspecifics and would wane over time if odor alone were applied. A repellent, however, could still be effective for short periods of time. Second, it is important to know how different age and sex classes respond to the odors being considered for application. The same odor may attract one sex or age and repel the other. For example, in muskrats (*Ondatra zibethica*) adults of both sexes avoided live traps scented with male musk during the breeding season, but immature muskrats did not discriminate between scented and unscented traps (Berk and Muller-Schwarze, 1984). Third, the odor has to be sufficiently species-specific to not attract other animal species, notably predators, that could interfere with the samples.

Once suitable odors have been found for practical application, it is important to emphasize that measures other than scenting should be taken. First, odors should be but one component of a comprehensive wildlife management program. Second, the odor should be formulated so that it is long-lasting in any weather, cost effective to manufacture, and easy to apply. This requires chemical identification of the active constituents, and embedding them into a slow-release matrix. Third, there should be a choice of scented and unscented sites. This is possible only in populations that are not too dense.

In summary, beaver pheromones hold promise as repellents that may prevent colonization of certain sites under well-understood ecological circumstances, but further studies are required to develop an efficient method of their application.

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FEEDING AND OVIPOSITION PREFERENCES OF
SWEET POTATO WEEVIL, *Cylas formicarius
elegantulus* (SUMMERS), ON STORAGE ROOTS
OF SWEET POTATO CULTIVARS WITH
DIFFERING SURFACE CHEMISTRIES

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Abstract—Cores from sweet potato [*Ipomoea batatas* (L.) Lam.] storage roots (Centennial, Jewel, Resisto, and Regal cultivars) were presented to sweet potato weevils [*Cylas formicarius elegantulus* (Summers) (Coleoptera; Curculionidae)] in multiple-choice, limited-choice, and no-choice bioassays. Centennial, a susceptible cultivar in field-plot experiments, was preferred for feeding and oviposition by female weevils in choice bioassays, and for oviposition in no-choice bioassays, compared to three other cultivars. Analysis of root surface chemistry showed a tentatively identified triterpenol acetate in Centennial, which was not found in the more resistant cultivars; another root surface component was found in higher concentrations in the more resistant cultivars.

Key Words—Sweet potato weevil, *Cylas formicarius elegantulus*, Coleoptera, Curculionidae, *Ipomoea batatas*, feeding, oviposition, host-plant preference, host-plant resistance, root surface chemistry.

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INTRODUCTION

A major constraint to sweet potato production worldwide is the sweet potato weevil, *Cylas* spp. (Edmond, 1971; Schalk and Jones, 1985; Sutherland, 1986), which causes substantial losses both in the field and during postharvest storage (Pillai and Nair, 1981; Talekar, 1982; Mullen, 1984). All parts of the plant are consumed, while eggs are laid singly in the storage roots (Reinhard, 1923; Cockerham et al., 1954). The development of insect-resistant sweet potato lines is seen as a viable component in the integrated management of this pest (Martin and Jones, 1986). Varying levels of resistance to *Cylas formicarius elegantulus* (Summers) have been shown for sweet potato cultivars in the field, in small field-plot tests (Cockerham and Deen, 1947; Cockerham and Harrison, 1952; Waddill and Conover, 1978; Rolston et al., 1979; Mullen et al., 1980b, 1981, 1982), and in laboratory bioassays (Mullen et al., 1980a; Barlow and Rolston, 1981; Nottingham et al., 1987). Resistance in the field studies may have been due to escape, for example, by having long thin storage roots set deep in the soil and scattered within growing hills (Cockerham et al., 1954; Jayaramaiah, 1975), tolerance (Velusamy and Heinrichs, 1986), antixenosis (nonpreference) (Kogan and Ortman, 1978; Singer, 1986), antibiosis (Painter, 1951), or a combination of any of these. The latter two types of plant resistance, which involve modification of insect behavior and metabolism, may have a chemical basis. Host-plant chemistry can potentially modify any stage of the weevil's host-location and utilization behavior from host finding (volatiles) through feeding and oviposition (surface chemicals) to larval development in the roots (internal root chemistry).

A methylene chloride root surface extract (Centennial cultivar) has been shown to stimulate oviposition in female weevils (Wilson et al., 1988). Two cultivars, Centennial and Jewel, known to be susceptible to the weevil in the field (Mullen et al., 1981) were used in these bioassays, along with two more recently developed cultivars having moderate levels of resistance, [Resisto (Jones et al., 1983) and Regal (Jones et al., 1985)]; methylene chloride extracts of these cultivars were analyzed using gas chromatography techniques (Son et al., 1987). The objective of this study was to compare the levels of feeding and oviposition on root cores with root surface chemistries in order to identify which chemical components might be responsible for the root extract modifying weevil behavior.

METHODS AND MATERIALS

Insect Rearing. Sweet potato weevils [*Cylas formicarius elegantulus* (Summers)] were reared in the laboratory on sweet potato storage roots in cages

held at 28°C and 65% relative humidity. Sweet potatoes used for rearing and bioassays were grown on the University of Georgia Horticulture Farm in 1986, harvested, cured, and stored at 15°C. Roots used in this study had been in storage five to seven months. Adult weevils were transferred to fresh potatoes every seven days; old potatoes were then incubated at room temperature until the new generation emerged. Emerging weevils were collected weekly and held in cages with potatoes until required for bioassays, between the ages of 20 and 35 days.

Bioassays. The bioassay used was an adaptation of one previously described by Mullen et al. (1980a) (Nottingham et al., 1987). Forty-eight male or female sweet potato weevils of known age were placed in plastic cages (32 × 24 × 10 cm) under experimental conditions (28°C, 90–100% relative humidity and 0:24 light–dark) 2 hr prior to treatments being introduced. Cores of sweet potato storage roots were taken with a cork borer (No. 11) and placed in 24-well Falcon tissue culture plates, such that the cores fitted in the wells (diam. 1.6 cm, depth 2 cm, area 2 cm²) with only the root periderm exposed. Four sweet potato cultivars were used: Centennial, Jewel, Resisto and Regal. These were presented in three different arrangements to the weevils:

(1) In the multiple-choice bioassay, the four cultivars were randomized within a 24-well plate (Nottingham et al., 1987). One plate was exposed to either 48 males or 48 females in each of four cages. (2) In the limited-choice bioassay, a choice of two cultivars, one cultivar in a block in each half of a 24-well plate, was presented to 48 females in each of six cages, such that all six combinations of the four cultivars occurred. (3) In the no-choice bioassay only one cultivar was presented in a 24-well plate in each of four cages to 48 females. For each type of bioassay, all weevils were taken from the same age batch and treatments were run at the same time.

After 24 hr, feeding punctures were counted. Beyond this time, the punctures tended to merge and reduce counting efficiency. After 48 hr, the plugged oviposition punctures were counted (Nottingham et al., 1987). Data were analyzed by analysis of variance, with orthogonal contrasts between treatments.

To obtain root periderm extracts, whole roots were submerged in a beaker containing methylene chloride and extracted for 9 min in an ultrasonic bath (Son et al., 1987). After solvent reduction on a roto-evaporator, a portion was taken down to dryness under nitrogen and treated with a silylation reagent, trimethylsilylimidazole-pyridine (1:3), for 1 hr at 76°C. After cooling, samples were analyzed by capillary gas chromatography on a 0.3-mm-ID × 25-m thin-coated SE-54 fused silica capillary column (temperature program 150–290°C at 4°C/min). Gas chromatograph profiles of all four cultivars were obtained, and extract component levels were calculated using an internal standard method (hexacosanol) and root surface area data, obtained by peeling periderm from storage roots and passing it through a LI-COR 3000 area meter.

RESULTS

In the multiple-choice bioassay, Centennial was preferred by female weevils for both feeding and oviposition, compared to the other three cultivars, which had similar levels of feeding and oviposition (Table 1). Males were found to have low levels of feeding on the roots (between 2.2 and 3.5 feeding punctures per 24 hr on average), compared to females, and no differences in feeding levels for males occurred between the four cultivars. Centennial was preferred by female weevils to each of the three other cultivars in the limited-choice bioassays for feeding and oviposition (Table 2). Jewel and Resisto were preferred to Regal for feeding and oviposition. A hierarchy of preference would place Centennial first, Jewel and Resisto intermediate, and Regal last, for both feeding and oviposition.

Female weevils had higher levels of feeding on Centennial and Regal than on Resisto in no-choice bioassays (Table 3). Weevils had higher levels of oviposition on Centennial than all three other cultivars in no-choice bioassays, while higher levels occurred on Resisto than Regal.

The gas chromatograph profiles of the root surface extract components of the four cultivars showed qualitative (Figure 1) and quantitative differences (Table 4). Centennial had a major component (peak 5), a triterpenol acetate, that was not present in the other three cultivars. Peak 2, which only occurred for Centennial, was the corresponding free alcohol of this compound (Son et al., 1987). Additionally, Centennial had a lower level of the component in peak 4 than the other three cultivars.

TABLE 1. FEEDING AND OVIPOSITION BY FEMALE *Cylas formicarius elegantulus*, 20-27 DAYS OLD, ON ROOT CORES OF FOUR SWEET POTATO CULTIVARS IN MULTIPLE-CHOICE BIOASSAY^a

	Feeding punctures (24 hr)	Eggs laid (48 hr)
Centennial	30.3 ^a ± 3.7	8.5 ^a ± 0.8
Jewel	16.6 ^b ± 2.1	5.3 ^b ± 0.6
Resisto	14.9 ^b ± 2.3	4.5 ^b ± 0.4
Regal	15.9 ^b ± 3.0	4.4 ^b ± 0.5

^aMeans (±S.E.) followed by the same letter are not significantly different ($P < 0.05$, ANOVA). $N = 24$ for all treatments.

TABLE 2. FEEDING AND OVIPOSITION BY FEMALE *Cylas formicarius elegantulus*, 25–35 DAYS OLD, ON ROOT CORES OF FOUR SWEET POTATO CULTIVARS IN SERIES OF PAIRED LIMITED-CHOICE BIOASSAYS^a

Pair	Feeding punctures (24 hr)	Eggs laid (48 hr)
Centennial	33.4 ^a ± 3.2	10.5 ^a ± 1.0
Jewel	24.3 ^b ± 2.3	7.0 ^b ± 0.8
Centennial	33.3 ^a ± 3.2	11.4 ^a ± 1.0
Resisto	23.8 ^b ± 1.8	5.3 ^b ± 0.6
Centennial	47.2 ^a ± 3.4	8.9 ^a ± 0.5
Regal	27.3 ^b ± 2.6	5.7 ^b ± 0.6
Jewel	40.1 ^a ± 2.6	7.8 ^a ± 0.7
Resisto	39.9 ^a ± 3.5	6.8 ^a ± 0.6
Jewel	39.9 ^a ± 3.9	6.2 ^a ± 0.8
Regal	23.8 ^b ± 2.7	4.2 ^b ± 0.4
Resisto	35.8 ^a ± 4.4	6.6 ^a ± 0.6
Regal	17.6 ^b ± 2.8	2.1 ^b ± 0.7

^aMeans (±SE), within each paired bioassay, followed by the same letter are not significantly different ($P < 0.05$, ANOVA). $N = 12$ for all treatments.

TABLE 3. FEEDING AND OVIPOSITION BY FEMALE *Cylas formicarius elegantulus*, 21–28 DAYS OLD, ON ROOT CORES OF FOUR SWEET POTATO CULTIVARS IN NO-CHOICE BIOASSAYS^a

Cultivar	Feeding punctures (24 hr)	Eggs laid (48 hr)
Centennial	41.1 ^a ± 7.0	11.5 ^a ± 0.9
Jewel	33.1 ^{ab} ± 2.8	6.4 ^{bc} ± 0.5
Resisto	23.7 ^b ± 4.0	7.5 ^b ± 0.7
Regal	37.1 ^a ± 3.1	5.7 ^c ± 0.5

^aMeans (±SE) followed by the same letter are not significantly different ($P < 0.05$, ANOVA). $N = 24$ for all treatments.

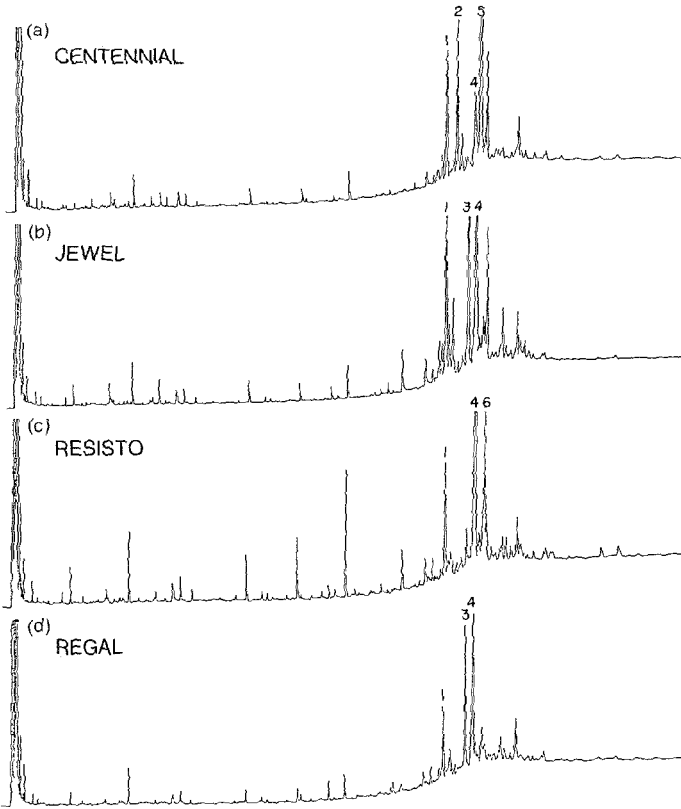


FIG. 1. GC profiles of root surface extracts: (a) Centennial, (b) Jewel, (c) Resisto, and (d) Regal.

DISCUSSION

Male weevils fed on the storage root cores to a lesser degree than females and did not show any feeding preferences between cultivars. This is in contrast to feeding on sweet potato leaves, where males and females were found to feed to an equal extent (Nottingham, et al., 1988). Females have to encounter the roots prior to oviposition, whereas males might obtain sufficient nutrients from the vines.

The relative levels of preference found for the four cultivars corresponded closely with results obtained in field plots (Mullen et al., 1985). Feeding and oviposition preference appeared to be closely related in choice bioassays. Barlow and Rolston (1981), in bioassays, showed that the ratio of oviposition to

TABLE 4. LEVELS OF SELECTED ROOT SURFACE COMPONENTS FROM FOUR SWEET POTATO CULTIVARS

Cultivar	Peak number ^a					
	1	2	3	4	5	6
	Relative retention time ^b					
	1.24	1.27	1.30	1.32	1.33	1.34
	Levels ($\mu\text{g}/\text{cm}^2 \times 100$) ^c					
Centennial	50.7	66.0	— ^d	26.7	367.7	— ^d
Jewel	35.5	— ^d	71.0	154.0	— ^d	— ^d
Resisto	21.8	— ^d	— ^d	153.0	— ^d	30.0
Regal	19.8	— ^d	64.0	138.0	— ^d	— ^d

^aNumbers correspond to peaks on chromatograms in Figure 1.

^bRelative to retention time of the internal standard, hexacosanol.

^cCalculated assuming chromatographic response equal to peak 5 isolate. Levels are averages of three analyses.

^dAbsent or below detection limits.

feeding punctures did not differ significantly among a range of cultivars. However, Regal had relatively high levels of feeding in no-choice bioassays, while being least preferred for oviposition. The differences in preference, which persisted in no-choice bioassays, may have a chemical basis, particularly for oviposition where differences were more clearly defined. Feeding and oviposition may be mediated by different host-plant chemicals. Centennial, the most preferred cultivar for oviposition, was found to have differences in its surface chemistry compared to the other three cultivars. The component in peak 5 (Figure 1), present in significant levels only in Centennial, was tentatively identified as a pentacyclic triterpene (molecular weight 468) and is currently being characterized (Son et al., 1987). This could potentially be an oviposition stimulant. Alternatively, Centennial has less of a component in peak 4 (molecular weight 498) (Figure 1), which could be inhibiting oviposition in the other cultivars.

The presence of an oviposition-stimulating substance in the root periderm has been demonstrated; Nottingham et al. (1987) showed that factors involved in cultivar preference and oviposition stimulation reside in the root periderm, while Wilson et al. (1988) reduced the oviposition-stimulating activity of roots (Centennial cultivar) by washing them in an ultrasonic bath of methylene chloride. When the methylene chloride root extract was applied back onto washed roots, oviposition behavior was restored, showing that behavioral activity

resided in the extract. Surface components of Centennial cultivar are currently being fractionated for further characterization, and behavioral activity of components will be determined in a root-core bioassay (Nottingham et al., 1987), concentrating initially on the identified triterpenol acetate.

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PRODUCTION OF ONION FLY¹ ATTRACTANTS AND OVIPOSITIONAL STIMULANTS BY BACTERIAL ISOLATES CULTURED ON ONION²

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Abstract—Decomposing onions at certain microbial successional stages produce potent volatile attractants and ovipositional stimulants of the onion fly, *Delia antiqua* (Diptera: Anthomyiidae). A reproducible source of these compounds was obtained by culturing *Erwinia carotovora* var. *carotovora* (EC) on sterile onion tissue. In laboratory choice tests, EC-inoculated onion was more attractive than *Klebsiella pneumoniae* (KP) cultured on onion, EC cultured on potato (a nonhost of onion fly), or the chemical synthetic baits dipropyl disulfide and an aqueous solution of 2-phenylethanol and pentanoic acid. Onion flies were mildly attracted to potato after inoculation with EC, but females did not accept EC-inoculated potato for oviposition. This work emphasizes that sources of semiochemicals may need to be defined micro-biologically as well as physically and chemically.

Key Words—Onion fly, *Delia antiqua*, Diptera, Anthomyiidae, *Erwinia carotovora* var. *carotovora*, *Klebsiella pneumoniae*, food attractant, ovipositional stimulant, dipropyl disulfide, 2-phenylethanol, pentanoic acid.

INTRODUCTION

Onion fly, *Delia antiqua* (Meigen) (Diptera: Anthomyiidae), is a major pest of commercial onions in the temperate northern hemisphere. Decomposing onions produce more potent onion fly attractants and ovipositional stimulants than do

¹ Diptera: Anthomyiidae.

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healthy or freshly cut onions (Dindonis and Miller, 1981a; Ishikawa et al., 1981), but only at certain successional stages of microbial and physical damage (Vernon et al., 1981; Miller et al., 1984). Using gas-liquid chromatography (GLC), Vernon et al. (1981) demonstrated that changes in onion fly attractancy were associated with qualitative and quantitative changes in the headspace chemical profile of onion as decomposition progressed.

Many factors influence volatile metabolite production in decomposing plant material. A complex interaction of environmental factors such as temperature, oxygen concentration, characteristics of the host plant, and presence of competing microorganisms and their metabolites may influence which organisms and volatile metabolites predominate. Under controlled growth conditions, microorganisms give reproducible headspace profiles that are often unique to a given genus, and, sometimes, species (Larsson et al., 1984). Indeed, using GLC for the qualitative and quantitative determination of volatile compounds has become an accepted technique in identifying anaerobic bacteria (Zechman and Labows, 1984) and some groups of aerobic bacteria (e.g., Lee et al, 1979). Bacteria infecting urine, meat, and other substrates are analyzed by direct headspace analysis of the sample or by subculturing the bacteria onto media that are selective or enriched in precursors to targeted metabolites (Larsson et al., 1984). Pure cultures may produce a distinctive series of volatile organics or a distinctive odor such as the grapelike 2-aminoacetophenone, a diagnostic marker for *Pseudomonas aeruginosa* (Cox and Parker, 1979).

The variable attractancy of decomposing onion has hindered work aimed at identifying potent onion fly attractants and ovipositional stimulants. This paper describes how a reproducible source of these compounds has been obtained by culturing bacterial isolates associated with onion decomposition on sterilized onion tissue.

METHODS AND MATERIALS

Insects. Parental *D. antiqua* stock was collected from commercial onion fields in Eaton Rapids, Michigan. Insects used were 5 to 7 generations removed from the field. Adults were fed water and a dry artificial diet (Ticheler, 1971) and housed as described by Schneider et al. (1983), except onion foliage in ovipositional dishes was replaced by green surrogate stems (Harris and Miller, 1983). Larvae were reared in sand-filled boxes provisioned with an excess of bisected onion bulbs.

All behavioral bioassays were conducted at $35 \pm 5\%$ relative humidity, $23 \pm 1^\circ\text{C}$, and in a 16 : 8 light-dark regime; alternating cool 55-W and warm 85-W white fluorescent bulbs were placed 10 cm above the cages. Three days before assaying, flies from the laboratory culture were placed in cylindrical,

metal-screened cages (42 cm diam. \times 46 cm). Between assays, flies were given food, water, and access to ovipositional dishes of freshly cut onion covered with moist sand. In the center of each dish we placed a 4-mm-diam. \times 9-cm-long surrogate onion stem. Before each assay, the water dish was placed in the center of the cage, but food and ovipositional dishes were removed.

Bacterial Cultures. *Erwinia carotovora* var. *carotovora* (EC) causes soft rot on a wide range of vegetables. Onion maggots moving through infested soil may spread the bacterium (Agrios, 1978). EC was isolated from damaged stored onions (Spartan Banner hybrid) potted in moist muck soil taken from a commercial onion field in East Lansing, Michigan. Damage was inflicted by 20 second-instar *D. antiqua* larvae taken from our laboratory culture and added to each potted bulb. After five days of larval feeding, 10 g of the decomposing onion tissue was homogenized with a sterile mortar and pestle in 25 ml sterile buffer (0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 , pH 7.0). For the isolation of soft-rot bacteria, dilutions of the homogenate were made in buffer and plated on a selective medium containing crystal violet and sodium polypectate (CVP) (Cuppels and Kelman, 1974). Plates were incubated overnight at $25 \pm 2^\circ\text{C}$.

Colonies of *Erwinia* spp. formed deep, cuplike depressions in the selective medium. They could be distinguished from pectolytic *Pseudomonas* spp., which form shallow, wider depressions (Cuppels and Kelman, 1974). A bacterial isolate of *Erwinia* sp. was obtained and designated as strain EC6. Isolate EC6 was identified using current taxonomic keys and texts (Lelliott, 1974; Kelman and Dickey, 1980).

Klebsiella pneumoniae (KP) had been stored at -65°C in 4% (v/v) DMSO in nutrient broth (0.3% beef extract and 0.5% peptone; Difco). The original strain, JM1, was isolated and identified as a predominant microorganism from decomposing onions that were five times more attractive in the field than fresh cut onions (Miller et al., 1984).

For use in experiments, colonies of both EC and KP were grown on Bacto nutrient agar (Difco) at $25 \pm 2^\circ\text{C}$. Stock cultures were maintained on nutrient agar in the refrigerator at $4-6^\circ\text{C}$.

Preparation of Axenic Onion and Potato. Axenic plant tissues were prepared from Russet Burbank potatoes and an undetermined cultivar of yellow onions from commercial storage. The outer, papery scales and upper and lower 2 cm of bulb were removed; potatoes were peeled. Plant tissues were soaked in 75% ethanol for 20 min and flame sterilized 1–2 min. The flamed outer 0.5 cm of potato and the flamed outer two onion scales were removed with sterilized instruments and discarded.

Two sizes of potato and onion tissue were cut. Squares of tissue measuring $3.5 \times 3.5 \times 0.5$ cm and $2 \times 2 \times 0.5$ cm were placed individually in polystyrene Petri dishes (diameters of 5.5 cm and 3.5 cm, respectively). Onion squares were cut only from the 2 to 3 outer scales. After sterile distilled water was

added to cover the dish bottom, tissues were incubated at $25 \pm 2^\circ\text{C}$. Because the concentration of sulfur- and nitrogen-containing compounds in onion are known to differ in the outer and inner scales (Freeman, 1975), only tissue taken from the same onion scale (or the same potato) was used within the same cage for bioassays.

Experiment 1: Attractancy of Axenic Onion. Approximately 100–125 flies of mixed sexes and ages were placed in each cylindrical cage. Wire screen baskets were hung in the tube traps of Weston and Miller (1985); all parts were autoclaved. Between 4 and 15 hr into photophase, treatments were enclosed individually in the wire baskets and assayed in choice tests by spacing traps equally along the periphery of cylindrical cages. After the bioassay, flies in each trap were sexed and counted.

In each of four cages, traps were baited with either a $3.5 \times 3.5 \times 0.5$ -cm axenic onion square cut immediately before assaying, cut axenic onion held for one, two, and three days before assaying, or a petri dish filled with sterilized, distilled water. At the time of cutting, two parallel grooves ca. 1 cm apart and 2.5 cm deep were cut into each square. Onion tissues were assayed in their original water-filled polystyrene petri dishes with the covers removed.

Experiment 2: Time course of Attractancy of Bacteria Cultured on Onion. To determine the minimal time needed to incubate soft rot bacteria on onion to obtain significant differences in onion fly attractancy, traps were baited with axenic onion inoculated with a bacterial isolate and incubated one, two, or three days before assaying. These treatments were compared to freshly cut axenic onion and a water control. To inoculate onion with bacteria, two parallel grooves ca. 1 cm apart and 2.5 mm deep were cut into each square. A loopful of 24-hr inoculum was placed in each groove. Tissues were incubated at $25 \pm 2^\circ\text{C}$. Experiments using EC and using KP were replicated seven times as described in experiment 1.

Experiment 3: Relative Attractancy of EC- and KP- inoculated Onion. To determine the relative attractancy of the two isolates cultured on onion, traps were baited with onion inoculated with EC, KP, or EC plus KP and incubated for three days at $25 \pm 2^\circ\text{C}$ before assaying along with freshly cut axenic onion. Onion was inoculated as described in experiment 2 with EC or KP inoculum placed in both grooves of a square or with EC inoculum in one groove and KP inoculum in the other. Eight replications were conducted as described in experiment 1.

Experiment 4: Attractancy of EC-Inoculated Host and Nonhost Plant. The volatile onion fly attractants produced by EC-inoculated onion may have been due to EC, regardless of the nutritive medium used, or to the interaction of EC with onion, the primary host plant of the onion fly. To determine the importance of the host plant in the production of attractants, EC was cultured on axenic onion and axenic potato, a nonhost plant of onion fly, as described in experi-

ment 2. Traps were baited with EC-inoculated onion, inoculated potato, axenic onion, axenic potato, and water control. Axenic onion was freshly cut; other treatments were incubated three days before assaying. This experiment was replicated seven times as described in experiment 1.

Experiment 5: Attractancy of EC-Inoculated Onion vs. Chemical Synthetics. The attractancy of onion inoculated with EC and incubated three days before assaying was compared to synthetic chemical attractants. One chemical attractant was a solution of 100 μ l 2-phenylethanol (2-PhEt) and 25 μ l pentanoic acid mixed in 50 ml distilled water. These chemicals were reported by Ishikawa et al. (1984) as *D. antiqua* attractants extracted from decomposing onions. This formulation was reported to give an optimal release rate for onion fly attractancy in the field (Ishikawa et al., 1984). Ten-milliliter glass beakers containing 10 ml of this aqueous solution were placed in traps immediately before testing.

A second chemical attractant was *n*-dipropyl disulfide (Pr_2S_2), a predominant volatile secondary metabolite from fresh onions (Block, 1985). For an optimal release rate (ca. 100 μ g/hr), 100 μ l Pr_2S_2 (purity 99%) was placed into size 3 BEEM (Ted Pella Co., Box 510, Tustin, California 92680) polyethylene enclosures (Dindonis and Miller, 1981b). One capsule per trap was used. To attain a stable release rate, filled capsules were held for 6 hr prior to assaying. The experiment was replicated four times as described in experiment 1.

Experiment 6: Oviposition on EC-Inoculated Host and Nonhost Plant. Ovipositional dishes (35 ml) were filled with sterilized sand moistened with sterilized, distilled water. A 4-mm-diam \times 9-cm-long surrogate onion stem stood vertically in the center of each dish. By spacing ovipositional dishes equally along the cage periphery, five different test materials were assayed simultaneously in cages containing six to eight gravid *D. antiqua* females. Treatments were assayed 8–14 hr into photophase, thus spanning the peak diurnal ovipositional period of *D. antiqua* (Havukkala and Miller, 1987).

In one ovipositional experiment, the 2.0 \times 2.0 \times 0.5-cm squares of plant tissue, along with associated water, were placed ca. 0.5 cm beneath the sand surface immediately before assaying. No plant material was placed in the cup designated as a water control. In a second experiment, to determine whether volatiles alone from test materials stimulated oviposition, Petri dishes containing the 3.5 \times 3.5 \times 0.5-cm squares of tissue and water were placed in sterilized glass quart canning jars. A GLC septum was inserted into each of two holes drilled into the jar lid. Teflon tubing (1 mm ID) was inserted into each septum. One tube passed through the screened cage wall and was threaded through a septum inserted into a hole in the side of the ovipositional cup. The tubing opened ca. 5 mm from the surrogate stem and ca. 1.25 cm below the sand surface. The second tube led from the jar lid to a small plastic manifold and then to the filter of a compressed air tank. Air carrying volatiles from the jars to the ovipositional cups flowed at 10 ml/min.

In both ovipositional experiments, the treatments used were onion and potato inoculated with EC and incubated for three days before assaying, axenic potato held three days before assaying, freshly cut axenic onion, and a water control. Preparation of treatments followed the procedure outlined in experiments 1 and 2. Both experiments were replicated four times.

RESULTS

Identification of Isolate EC6. Bacterial cells of EC6 were motile, straight rods occurring primarily singly, but sometimes in pairs or short chains. This isolate was gramnegative, facultatively anaerobic, oxidase negative, phosphatase negative, reduced nitrate, did not produce reducing substances from sucrose, and utilized Simmon's sodium citrate. Growth occurred at 36°C and in 5% NaCl. No special growth factors were required. Colonies on yeast extract-dextrose-CaCO₂ agar (YDC) were creamy white with no diffusible pink or blue pigments; colonies on nutrient agar were translucent white with no yellow pigments. Inoculation on sterile potato disks caused soft rot. Based on these results and the characteristic growth on CVP medium, EC6 was identified as a strain of *Erwinia carotovora* var. *carotovora*.

Experiment 1: Attractancy of Axenic Onion. Sterilized onion tissues incubated one day or more before the assay lost activity (Figure 1); therefore, sterilized onions used as controls in the remaining bioassays were cut immediately before assaying.

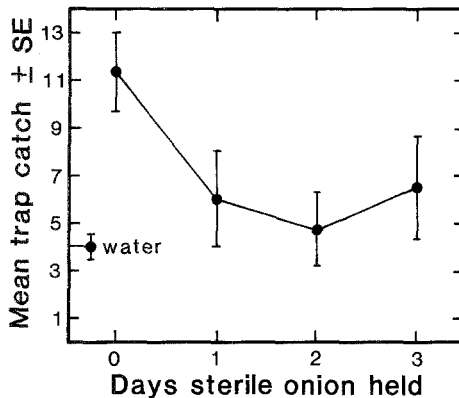


FIG. 1. Onion fly attractancy as influenced by time sterilized onion tissue was held before assaying. Orthogonal contrasts after ANOVA: freshly cut onion vs. onion incubated 1, 2, and 3 days ($P < 0.05$); freshly cut onion vs. water control ($P < 0.05$); and water control vs. onion incubated 1, 2, and 3 days ($P > 0.20$).

Experiment 2: Time Course of Attractancy of Bacteria Cultured on Onion. Attractancy of EC-inoculated onion increased with incubation times from one to three days (Figure 2A). Onion inoculated and incubated two or three days caught significantly more flies than either noninoculated onion or the water control. A similar pattern of increasing attractancy with incubation times from one to three days was seen for KP-inoculated onion tissue (Figure 2B). A three-day incubation period was judged sufficient to obtain significant differences in attractancy between bacterially inoculated onion and sterile onion.

Experiment 3: Relative Attractancy of EC- and KP-Inoculated Onion. Traps baited with onion inoculated with EC caught significantly more flies than those baited with KP-inoculated onion (Figure 3). Onion tissue inoculated with both bacteria did not catch significantly more flies than onion inoculated with

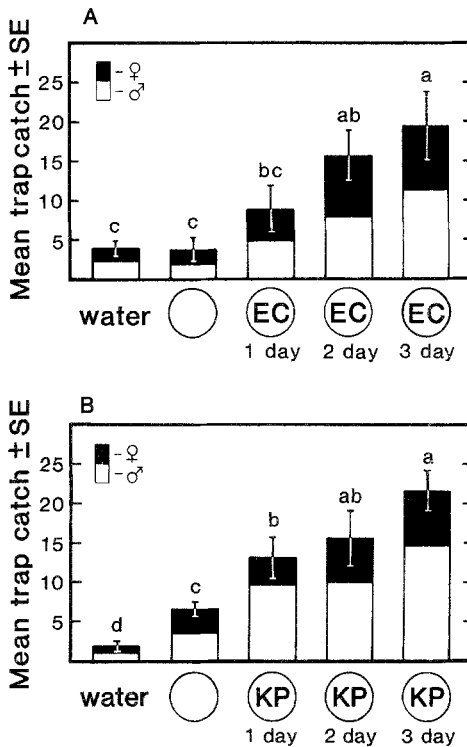


FIG. 2. Influence of incubation time on onion fly attractancy of onion inoculated with bacterial isolates. Sterilized onion tissue (○) inoculated with EC (A) or with KP (B) and incubated 1, 2, or 3, days before the bioassay. Treatments marked by different letters are significantly different (Tukey's HSD all-pairwise comparisons after ANOVA; $P < 0.05$ for A; $P < 0.10$ for B).

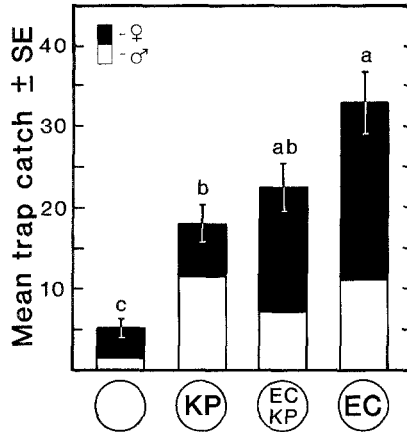


FIG. 3. Relative onion fly attractancy of EC and/or KP inoculated on sterilized onion tissue (○) and incubated 3 days before the bioassay. Treatments marked by different letters are significantly different (Tukey's HSD all-pairwise comparisons after ANOVA; $P < 0.05$).

either bacterial isolate alone ($P > 0.20$). Regardless of the bacterial isolate used, all inoculated onion baits caught significantly more flies than traps baited with noninoculated onion. The remaining experiments focused on the bacterial isolate EC.

Experiment 4: Attractancy of EC-Inoculated Host and Nonhost Plant. A higher mean trap catch was attained when onion rather than potato was inoculated with EC (Figure 4). Traps baited with EC-inoculated potato did catch significantly more flies than those baited with noninoculated potato.

Experiment 5: Attractancy of EC-Inoculated Onion vs. Chemical Synthetics. Traps baited with EC-inoculated onion caught significantly more flies than any other bait used (Figure 5). The mean fly catches obtained with traps baited with either chemical attractant or with noninoculated onion were all low and did not differ significantly from each other ($P > 0.20$). All baits tested caught significantly more flies than the water control, except Pr_2S_2 ($P > 0.20$).

Experiment 6: Oviposition on EC-Inoculated Host and Nonhost Plant. When test materials were placed directly in the ovipositional dishes, EC-inoculated onion received significantly more eggs than any other treatment (Table 1, experiment A). Freshly cut, sterilized onion received fewer eggs than EC-inoculated onion but more eggs than all other treatments. No other significant differences were found ($P > 0.20$).

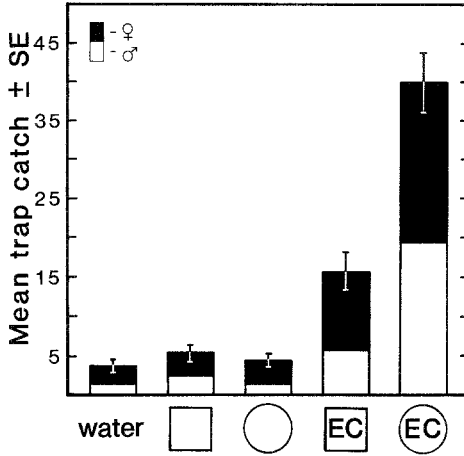


FIG. 4. Production of onion fly attractants by the interaction of EC on onion fly host and nonhost tissue. Sterilized onion tissue (○) and potato tissue (□) inoculated with EC and incubated 3 days before the bioassay. Statistically significant interaction between plant tissue used and presence or absence of EC [two-way ANOVA of data transformed to $(X \pm 0.5)^{1/2}$; $p < 0.01$]. Orthogonal contrasts: potato vs. EC-inoculated potato ($P < 0.01$); onion vs. EC-inoculated onion ($P < 0.01$); EC-inoculated potato vs. EC-inoculated onion ($P < 0.01$); potato vs. onion ($P > 0.20$).

When volatiles from these treatments were pumped into the dishes, EC-inoculated onion received significantly more eggs than any other treatment group (Table 1 experiment B). No other differences were found among the remaining treatments ($P > 0.20$), all of which received very few eggs.

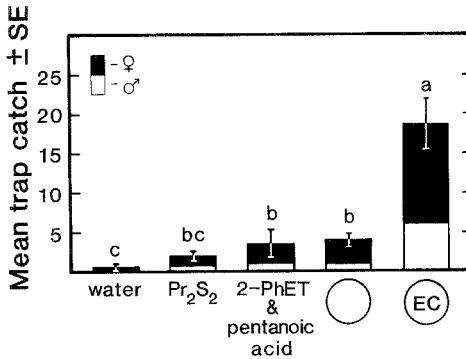


FIG. 5. Comparison of chemical synthetics and EC-inoculated onion as onion fly attractants. Symbols and statistics as in Figure 3.

TABLE 1. MEAN NUMBER OF EGGS LAID^a BY *D. antiqua* IN RESPONSE TO EC CULTURED ON HOST AND NONHOST PLANT TISSUE

Treatment	Experiment ^b	
	A	B
Ec-inoculated onion	73.5 ^a ± 23.87	47.5 ^a ± 10.54
Noninoculated onion	12.5 ^b ± 2.96	1.0 ^b ± 0.56
EC-inoculated potato	1.0 ^c ± 1.00	1.0 ^b ± 0.41
Noninoculated potato	0.0 ^c	0.0 ^b
Water control	0.0 ^c	0.0 ^b

^aMeans ± SE followed by the same letter within a column are not significantly different [one-way ANOVA of data transformed to $(X + 0.5)^{1/2}$ followed by Tukey's all-pairwise comparisons (Gill, 1978); $P < 0.05$].

^bTreatments presented in ovipositional dishes (experiment A) or as volatiles pumped into ovipositional dishes (experiment B).

DISCUSSION

The unstable sulfenic acids released from cut onion degrade to form various sulfur-containing volatiles that, in large part, give onion its characteristic odor and taste (Block, 1985). A number of these volatiles, (e.g., dipropyl disulfide) are known to attract and/or stimulate oviposition by the onion fly (Matsumoto and Thorsteinson, 1968; Vernon et al., 1978). As the volatiles from damaged onion cells are exhausted, the attractancy of freshly cut sterilized onion would be expected to decrease; hence, freshly cut sterilized onion was chosen as an appropriate control for assays testing the effects of culturing bacteria on sterilized onion tissue.

The interaction of EC and of KP with onion tissue increased *D. antiqua* trap catches (Figure 2) and oviposition (Table 1). Behavioral activity in response to EC- or KP-inoculated onion increased with time (Figure 2), probably due to a rise in volatile release. As the bacterial population increases, a concomitant increase in amounts of pectolytic and cellulolytic enzymes would be expected (Starr and Chatterjee, 1972). Qualitative changes in volatiles also would be expected as the chemical composition of the host changes and bacteria exhaust available nutrients.

Human olfaction detects little difference between sterilized and EC-inoculated potato tissue. Indeed, many vegetables infected with *E. carotovora* var. *carotovora* alone do not release the unpleasant odors commonly associated with decomposing plants. Saprophytic bacteria that colonize plant tissue after pathogenic infection generally are responsible for the foul odor. Two notable exceptions are crucifers and alliums, both of which release unpleasant sulfurous odors when inoculated with the soft-rot pathogen (Agrios, 1978). EC-inoculated potato

may release simple volatile metabolites not particularly odoriferous but nevertheless mildly attractive to onion fly, e.g., short-chain alcohols and hydrocarbons (Stotzky and Schenck, 1976).

The aqueous solution of 2-PhEt and pentanoic acid reported by Ishikawa et al. (1984) to be a potent attractant of *D. antiqua* was much less attractive than EC-inoculated onion tissue (Figure 5), suggesting that attractants associated with decomposing onions remain to be identified. Indeed, the low fly catches obtained with 2-PhEt/pentanoic acid as well as with Pr₂S₂ demonstrate that currently known synthetic attractants do not closely simulate fresh or maximally attractive decomposing onion. Perhaps decomposing onions used as positive controls in the studies emphasizing the potency of 2-PhEt/pentanoic acid as attractants for *D. antiqua* were inadequate (Ishikawa et al., 1984).

Even though they were significantly attracted, female onion flies did not accept EC-inoculated potato as an ovipositional site (Table 1). For *D. antiqua*, oviposition is a specific behavioral response restricted to stimuli from *Allium cepa* and its closest relatives. On the other hand, attractancy can include both host-finding and food-finding. Thus, onion flies may be attracted to and feed on decomposing nonhost plants (Miller et al., 1984) but may not accept them for oviposition.

The most notable points made here are: (1) that a source of consistent highly active *D. antiqua* attractants has been found (this advance should greatly facilitate chemical identification of these attractants), and (2) sources of behavior-modifying stimuli may need to be defined microbiologically as well as physically and chemically.

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INHIBITION AND RECOVERY OF CUCUMBER ROOTS GIVEN MULTIPLE TREATMENTS OF FERULIC ACID IN NUTRIENT CULTURE¹

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Abstract—Ferulic acid, a frequently cited allelopathic agent, inhibited photosynthesis, leaf expansion, and root elongation of cucumber seedlings grown in aerated nutrient cultures in a growth chamber. Other effects were a reduction in the proportion of radioactivity fixed by photosynthesis translocated to roots, a stimulation in secondary root initiation, and an increase in root–shoot ratios. Inhibition of leaf expansion and root elongation induced by multiple ferulic acid treatments was rapidly lost once ferulic acid was removed from the root environment. The changes in general root morphology, i.e., average root length and root number, associated with ferulic acid treatments, were partially reversed or not affected when ferulic acid was removed from the root environment.

Key Words—Allelopathy, ferulic acid, leaf expansion, root elongation, carbon allocation, *Cucumis sativus*, cucumber.

INTRODUCTION

Ferulic acid, a frequently cited allelopathic compound (Rice, 1984), has been shown to inhibit germination, radicle elongation, leaf area expansion, and dry matter accumulation of shoots and roots (Patterson, 1981; Einhellig et al., 1985; Waters and Blum, 1987; Blum et al., 1987). Effects of ferulic acid are rapidly

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lost, however, once it is removed from the root environment (Blum et al., 1985a; Balke, 1985; Waters and Blum, 1987). Exposure of plant roots to ferulic acid reduces plant water utilization (Blum and Dalton, 1985; Blum et al., 1985a), modifies turgor and osmotic pressure, closes stomates (Einhellig et al., 1985), and reduces rates of photosynthesis (Patterson, 1981) and ion uptake (Balke, 1985).

Shoots and roots are in many ways complementary in function because both supply required resources to the whole plant. This makes the two organs dependent on each other and suggests that their growth rates are under mutual control (Brouwer, 1983). The underlying physiological mechanisms, however, are poorly understood. Information in the literature on the effects of ferulic acid on shoots of a variety of species is extensive (Rice, 1984), while information on the effects of ferulic acid on roots is limited. The objective of this study was to determine the effects of ferulic acid on roots and root-shoot relationships. Cucumber (*Cucumis sativus*) was chosen as the model species because of its sensitivity to ferulic acid, its rapid growth rates, and its predictable behavior (Blum and Dalton, 1985; Blum et al., 1985a,b). The present report represents an effort to refine methodology that will aid in the understanding of allelopathic interactions.

METHODS AND MATERIALS

General Aspects. Cucumber seeds (*Cucumis sativus* cv. Early Green Cluster; Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated in the dark at 25°C in trays containing vermiculite and a modified nutrient solution (see below). After 72 hr in the dark, the seedlings were exposed to light [photosynthetic photon flux density (PPFD) 642 $\mu\text{mol m}^2/\text{sec}$] for 10 hr and then transferred to glass jars containing 430 ml nutrient solution. Seedlings were suspended in the nutrient solution by a foam collar through a hole in the cap of the jars. Light was excluded from the jar with foil. Nutrient solutions containing seedling roots were aerated continuously 48 hr after transplanting.

Plants were grown in a growth chamber of the Southeastern Plant Environmental Laboratory (North Carolina State University Phytotron) with 25°C day/night temperature, a 10-hr photoperiod (PPFD of 642 $\mu\text{mol m}^2/\text{sec}$ provided by incandescent and cool white fluorescent bulbs at an input wattage ratio of 3:10, respectively), and a relative humidity > 80%. Phytotron nutrient solution (Downs and Thomas, 1983) without sodium fluorescein and supplemented with 606 mg KNO_3 and 84 mg K_2HOP_4 per liter was used throughout the study. Deionized water was added twice a day to each jar to compensate for evapotranspiration. Nutrient solutions with or without ferulic acid (Sigma Chemical

Company, St. Louis, Missouri) were changed every other day. The initial pH of all solutions was 5.5.

Leaf length and width were recorded just before solution changes and/or at final harvest. Shoot and root weights were measured at various intervals and/or at final harvest. Roots sampled to determine root number and length were stored in a 70% EtOH solution.

Leaf Areas. Leaf length, width, and area were determined (Li-Cor model 3000 Leaf Area Meter, Lambda Instruments Co.) at various ages ranging from 9 to 20 days from seed. These data were used to develop the following model:

$$\text{Leaf area} = -4.77 + 0.00916 (LW); r^2 = 0.97; N = 95$$

where leaf area is in square cm and LW equals leaf length \times width in mm. This model was used to determine leaf area for all experiments. The areas of the cotyledons were not included in the estimates of total leaf area per plant.

Effects of Ferulic Acid on Leaf Areas and Roots. Seedlings were treated with 0, 0.125, 0.25, or 0.5 mM ferulic acid (six seedlings per concentration) in fresh nutrient solution every other day starting with day 7. Leaf measurements were made and plants were harvested 15 days from seed. Root systems were separated from the shoot and placed into bottles containing 70% EtOH. To obtain root measurements: (1) roots were floated in a pan of tap water, (2) the primary root was identified, (3) all secondary roots were cut from the primary root, and (4) root length of both the primary and the secondary roots was determined. The number of secondary roots was also recorded. In addition, the number of tertiary roots was counted on 10 randomly selected secondary root sections of > 10 cm length.

Dimension Analysis. Seedlings were treated with 0, 0.125, 0.25, or 0.5 mM ferulic acid (12 seedlings per concentration) in fresh nutrient solution every other day starting on day 8 from seed. Root and shoot dry weight, leaf area, root number, and root length were determined when seedlings were 16 days old. Leaf areas were determined for all seedlings, but dry weights and root measurements were made on different sets of seedlings (six seedlings per concentration per set).

Effects of Ferulic Acid on Carbon Allocation to Roots. Seedlings were treated with 0, 0.25, or 0.5 mM ferulic acid in fresh nutrient solution every other day starting on day 8. Immediately after solutions were changed on day 14, seedlings were placed into Plexiglas chambers (18 cm high, 19.5 cm diameter) for exposure to $^{14}\text{CO}_2$. Six seedlings (one per chamber) were exposed to $^{14}\text{CO}_2$ each exposure. Exposures were conducted between 1200 and 1600 hr on two consecutive days, and two sets of seedlings were exposed each day. Temperature in the exposure chambers ranged from 25 to 30°C and PPFD was approximately 513 $\mu\text{mol cm}^2/\text{sec}$. $^{14}\text{CO}_2$ was generated by the action of 1 N

HCl on [^{14}C]Na $_2$ CO $_3$ in aqueous solution (4 μCi of ^{14}C in 20 μl of 1 N NaOH) to generate a radioactivity of 2.97×10^7 dpm/liter of chamber atmosphere. After 15 min, residual $^{14}\text{CO}_2$ was captured in 3 N NaOH by means of a circulating pump. Thirty minutes later, seedlings were removed from the chambers. Half the plants were harvested immediately, and the other half were harvested 24 hr later. On harvest, plants were cut into sections (leaves, stem, and roots), weighed, and respective sections were placed into separate scintillation vials. Sections were oven-dried (40°C) and chopped, and the dry weight in each vial was calculated using the appropriate fresh-dry weight ratios determined from sections of unlabeled seedlings. To bleach the tissue, a 30% hydrogen peroxide solution (0.5 ml/50 mg tissue) was added to each vial, and vials were placed under a light bank until the samples were dry. EtOH (2 ml, 95%) was added to each vial, and the capped vials were incubated at 40–50°C overnight (Shann and Blum, 1987a). Scintiverse LC (Fisher Scientific Co., Springfield, New Jersey) cocktail was added, and the radioactivity was determined by liquid scintillation spectrophotometry (Beckman LS 7000). The counting efficiency of radioactivity was 70%. Background radiation of unlabeled tissue averaged 30 cpm. The experimental design was: three ferulic acid concentrations, two harvests per exposure, and four exposures, totaling 24 seedlings.

Recovery after Ferulic Acid Treatments. Seedlings were treated with 0 or 0.5 mM ferulic acid in fresh nutrient solution starting on day 7 and given two (days 7 and 9), three (days 7, 9, 11), or four (days 7, 9, 11, 13) ferulic acid treatments. Solutions in jars were changed every other day. Four seedlings were harvested every other day for the control and the four-times-treated seedlings. For the remaining treatments, seedlings were harvested every other day starting 48 hr after the last ferulic acid treatment. Mean absolute rates of leaf expansion were determined by the following equation:

$$AGR = \text{leaf area at time}_x - \text{leaf area at time}_{x-1}$$

where AGR is in square cm per two days. Mean relative rates of leaf expansion were determined as follows:

$$RGR = \log(\text{leaf area at time}_x) - \log(\text{leaf area at time}_{x-1})$$

where RGR is in square cm per square cm per two days. For additional details about growth analysis equations see Evans (1972).

Data Analyses. Log-transformed and untransformed data were analyzed using Statistical Analysis System (SAS Institute Inc., Cary, North Carolina) programs for analysis of variance and regressions. Logarithmic transformations were used to stabilize the variance of the radioactivity data because the error is inversely proportional to the magnitude of the mean count. Linear, quadratic, and cubic equations were fitted to data. Least significant differences (LSD 0.05)

were calculated as a measure of experimental precision. Inferences are based on the appropriate analysis of variance or regression analysis ($\alpha \leq 0.05$).

RESULTS

Effects of Ferulic Acid on Leaf Areas and Roots. As ferulic acid treatment concentrations were increased, concurrent decreases were obtained for leaf area [area (cm²) = 288.20 - 178.57 (conc.); $r^2 = 0.38$; where conc. is in mM], root length of primary and secondary roots [length (cm) = 678.14 - 563.02 (conc.); $r^2 = 0.74$], average root length of primary and secondary roots [average length (cm) = 37.39 - 37.71 (conc.); $r^2 = 0.83$], and the number of tertiary roots [number = 243.21 - 220.70 (conc.); $r^2 = 0.67$]. All such relationships were linear, and higher-order terms were not significant. The number of primary and secondary roots and the frequency (number/cm) of tertiary roots were not modified by multiple ferulic acid treatments. One reason for the low r^2 values of some of these models can be seen in Table 1 where, for example, leaf area was only reduced substantially by the 0.5 mM ferulic acid treatment.

Dimension Analysis. Leaf area, shoot and root weight, and total and average length of primary and secondary roots at final harvest were suppressed by multiple treatments of ferulic acid (Tables 2 and 3). The root-shoot ratio and the number of roots (primary and secondary) increased with increasing ferulic acid treatment concentrations.

Models developed with leaf area as the independent variable and dry weight, total and average root length, or root number as dependent variables demonstrated that most variables were directly related to leaf area (Table 4).

TABLE 1. EFFECTS OF MULTIPLE TREATMENTS OF FERULIC ACID ON CUCUMBER SEEDLINGS GROWN IN AERATED NUTRIENT SOLUTION

Concentration of ferulic acid (mM)	Leaf area (cm ²)	Root length (cm)*	Number of roots	Average root length (cm)*	Tertiary roots per cm	Number of tertiary roots
0	269.74 ^{a**}	653.63 ^a	17.67 ^a	37.02 ^a	0.36 ^a	235.97 ^a
0.125	287.30 ^a	626.65 ^{ab}	18.83 ^a	33.61 ^a	0.36 ^a	222.88 ^{ab}
0.25	248.35 ^a	558.08 ^b	20.67 ^a	27.27 ^b	0.34 ^a	191.62 ^b
0.5	191.16 ^b	381.57 ^c	21.00 ^a	18.64 ^c	0.34 ^a	129.25 ^c
LSD _{0.05}	52.59	77.30	4.22	4.07	0.04	37.14

*Values based on primary and secondary roots.

**Column means followed by the same letter are not significantly different.

TABLE 2. EFFECTS OF MULTIPLE TREATMENTS OF FERULIC ACID ON CUCUMBER SEEDLINGS GROWN IN AERATED NUTRIENT SOLUTION

Concentration of ferulic acid (mM)	Leaf area (cm ²)	Shoot dry weight (g)	Root dry weight (g)	Root-shoot ratio	Root length (cm)*	Number of roots	Average root length (cm)*
0	257.88 ^{ab**}	0.96 ^c	0.18 ^a	0.18 ^a	465.92 ^a	19.17 ^a	24.42 ^a
0.125	277.45 ^a	0.92 ^{ab}	0.18 ^a	0.19 ^a	510.42 ^a	24.83 ^b	20.83 ^b
0.25	240.70 ^b	0.85 ^b	0.16 ^a	0.19 ^a	457.57 ^a	32.33 ^c	14.10 ^c
0.5	154.32 ^c	0.55 ^c	0.12 ^b	0.23 ^b	341.67 ^b	42.67 ^d	8.05 ^d
LSD _{0.05}	28.00	0.09	0.03	0.02	82.19	4.46	3.31

* Values based on primary and secondary roots.

** Column means followed by the same letter are not significantly different.

TABLE 3. PARTIAL REGRESSION COEFFICIENTS AND r^2 VALUES FOR LEAF AREA, DRY WEIGHT, ROOT-SHOOT RATIO, ROOT LENGTH, NUMBER OF ROOTS, AND AVERAGE ROOT LENGTH OF CUCUMBER SEEDLINGS GIVEN MULTIPLE FERULIC ACID TREATMENTS^a

Variable	Intercept	Linear	Quadratic	r^2
Leaf area (cm ²)	262.13	115.08	-667.09	0.67
Shoot weight (g)	0.96	-0.07	-1.48	0.84
Root weight (g)	0.18	-0.11		0.44
Root/shoot ratio	0.18	0.084		0.47
Root length (cm) ^b	473.60	263.69	-1065.38	0.48
Number of roots ^b	19.37	47.47		0.87
Average root length (cm) ^b	24.19	-33.56		0.84

^aIndependent variable = concentration; 0, 0.125, 0.25, and 0.5 mM.

^bValues based on primary and secondary roots.

Log transformation of variables (i.e., dependent and/or independent) did not improve the r^2 values of models based on nontransformed data. Root dry weight could be estimated from shoot dry weight by a linear model (Table 4).

Effects of Ferulic Acid on Carbon Allocation to Roots. Growth of seedlings exposed to ¹⁴CO₂ was suppressed by ferulic acid treatments (Table 5). Seedling photosynthesis (based on total seedling radioactivity immediately after the ¹⁴CO₂ exposure) was inhibited by multiple ferulic acid treatments (log radioactivity (cpm) = 11.46 - 0.98 (conc.); r^2 = 0.31; where conc. is in mM; also see Table 6). Radioactivity of seedlings was 101,838, 71,381 or 63,371 cpm for the 0, 0.25, or 0.5 mM ferulic acid treatments, respectively. This amounted to a 30 or 38% reduction in radioactivity for the 0.25 or 0.5 mM treatments, respectively.

TABLE 4. PARTIAL REGRESSION COEFFICIENTS AND r^2 VALUES FOR DRY WEIGHT, ROOT LENGTH, NUMBER OF ROOTS, AND AVERAGE ROOT LENGTH^a

Variable	Intercept	Linear	Quadratic	r^2
Shoot weight (g)	0.16	0.003		0.92
Root weight (g)	0.06	0.0004		0.62
Root weight II (g)	0.03	0.15		0.75
Root length (cm)	130.05	1.38		0.67
Number of roots	102.76	-0.57	0.001	0.32
Average root length (cm)	-36.95	0.40	-0.0007	0.48

^aIndependent variable = leaf area (cm²) for all but root weight II where the independent variable = shoot dry weight (g).

TABLE 5. EFFECTS OF MULTIPLE FERULIC ACID TREATMENTS ON LEAF AREA AND DRY WEIGHT OF CUCUMBER SEEDLINGS EXPOSED TO $^{14}\text{CO}_2$

Concentration of ferulic acid (mM)	Leaf area (cm ²)	Shoot dry weight (g)	Root dry weight (g)	Seedling dry weight (g)
Initial values*				
0	150.88 ^{a**}	0.77 ^a	0.10 ^a	0.87 ^a
0.25	128.05 ^{ab}	0.69 ^{ab}	0.09 ^a	0.78 ^a
0.5	107.02 ^b	5.41 ^b	0.07 ^b	0.57 ^b
LSD _{0.05}	30.82	0.16	0.02	0.18
Final values***				
0	182.21 ^a	1.09 ^a	0.14 ^a	1.23 ^a
0.25	132.24 ^b	0.94 ^b	0.13 ^a	1.07 ^a
0.5	112.80 ^b	0.68	0.09 ^b	0.77 ^b
LSD _{0.05}	31.89	0.14	0.03	0.17

* Immediately after the $^{14}\text{CO}_2$ exposure.

** Column means followed by the same letter are not significantly different.

*** 24 hr after the $^{14}\text{CO}_2$ exposure.

TABLE 6. EFFECTS OF MULTIPLE FERULIC ACID TREATMENTS ON DISTRIBUTION OF RADIOACTIVITY IN CUCUMBER SEEDLINGS

Concentration of ferulic acid (mM)	Log transformed cpm values			Percent of radioactivity in roots
	Shoot	Root	Seedling	
Initial values*				
0	11.48 ^{a**}	7.58 ^a	11.50 ^a	1.98 ^a
0.25	11.13 ^{ab}	7.02 ^b	11.15 ^{ab}	1.67 ^b
0.5	10.99 ^b	6.88 ^b	11.01 ^b	1.62 ^c
LSD _{0.05}	0.44	0.56	0.44	0.004
Final values***				
0	11.33 ^a	9.21 ^a	11.44 ^a	10.85 ^a
0.25	11.01 ^b	8.69 ^b	11.11 ^b	8.91 ^b
0.5	10.73 ^b	8.18 ^c	10.81 ^c	7.34 ^c
LSD _{0.05}	0.30	0.41	0.30	0.02

* Immediately after $^{14}\text{CO}_2$ exposure.

** Column means followed by the same letter are not significantly different.

*** 24 hr after $^{14}\text{CO}_2$ exposure.

Twenty-four hours after the $^{14}\text{CO}_2$ exposure, radioactivity of shoots was 84,428, 62,627 or 46,527 cpm and of roots was 10,325, 6156, or 3725 cpm for the 0, 0.25, or 0.5 mM ferulic acid treatments, respectively. Models for shoot, root, and seedling log cpm were as follows: (1) shoot log cpm = $11.32 - 1.19 (\text{conc.})$; $r^2 = 0.60$ where conc. is in mM; (2) root log cpm = $9.2 - 2.04 (\text{conc.})$; $r^2 = 0.70$; and (3) seedling log cpm = $11.44 - 1.27 (\text{conc.})$; $r^2 = 0.63$. The radioactivity of roots expressed as a percentage of total seedling radioactivity [$\% \text{ root cpm} = 0.1079 - 0.070 (\text{conc.})$; $r^2 = 0.50$] was reduced by 18 or 32% for the 0.25 or 0.5 mM ferulic acid treatments, respectively (Table 6).

Recovery after Ferulic Acid Treatments. Significant differences for leaf area and root length due to ferulic acid treatments were lost almost immediately after seedlings were removed from ferulic acid solutions (Table 7). For average root length, a partial recovery occurred after roots were removed from ferulic acid, while the effects of ferulic acid treatments on root number persisted after seedlings were removed from ferulic acid solutions. Recovery of absolute rates of leaf expansion occurred within 48 hr after seedlings were removed from ferulic acid solutions (Table 8). Relative rates of leaf expansion were stimulated 48 hr after the seedlings were removed from ferulic acid solutions.

DISCUSSION

Consistent measurable effects of ferulic acid on shoots and roots of cucumber seedlings occurred at ≥ 0.25 mM. One of the documented effects of ferulic acid is the inhibition of leaf expansion (Blum and Dalton, 1985; Blum et al. 1985a,b; Waters and Blum, 1987). Effects of ferulic acid on root elongation, however, have not been well documented. The previously reported inhibition of radicle growth (Blum et al., 1984), the inhibition of root elongation reported here, and the subsequent recovery of both following removal from ferulic acid solutions suggest that cell elongation (i.e., cell expansion) in roots is also impacted by ferulic acid. Cell division may also be affected by ferulic acid, but this has not been determined. Roots of seedlings treated with ferulic acid (≥ 0.25 mM) appeared to be much thinner, tan in color, and frequently with enlarged or deformed root tips. Control roots were creamy white in color.

Ferulic acid also directly or indirectly stimulated the initiation of secondary roots in two of three experiments. In one experiment the average number of roots increased, but this increase was not significant. Thus, ferulic acid-treated root systems were not only shorter and more delicate but also more branched. The nature of this difference is illustrated by average root length, which was

TABLE 7. LEAF AREA, ROOT LENGTH, NUMBER OF ROOTS, AND AVERAGE ROOT LENGTH AT VARIOUS TIMES AFTER MULTIPLE 0.5 mM FERULIC ACID TREATMENTS^a

Days of treatments	Seedling age (days)					Seedling age (days)				
	7	9	11	13	15	7	9	11	13	15
Leaf area (cm ²)	21.13	48.15 ^a	94.79 ^a	170.58 ^a	270.18 ^a	179.00	259.98 ^a	299.50 ^a	401.75 ^a	468.88 ^a
7, 9				130.72 ^b	253.54 ^a				393.38 ^a	556.62 ^a
7, 9, 11					225.06 ^a					470.38 ^a
7, 9, 11, 13		36.63 ^b	71.30 ^b	125.36 ^b	165.44 ^b		166.38 ^b	267.62 ^a	252.12 ^b	341.75 ^b
LSD _{0.05}		8.56	21.05	33.91	56.80		35.85	91.30	95.89	127.50
Number of roots	20	18.25 ^a	17.75 ^a	19.25 ^a	18.00 ^a	Average root length (cm)				
7, 9				25.75 ^a	29.00 ^b	8.95	14.48 ^a	16.94 ^a	21.29 ^a	26.35 ^a
7, 9, 11					34.50 ^b				15.33 ^b	19.27 ^b
7, 9, 11, 13		16.25 ^a	26.50 ^b	24.25 ^a	35.75 ^b		10.42 ^b	10.27 ^b	10.43 ^c	13.74 ^c
LSD _{0.05}		5.63	7.47	6.88	7.62		3.26	2.36	3.46	2.91

^aColumn means followed by the same letters are not significantly different.

TABLE 8. RECOVERY OF LEAF GROWTH AFTER MULTIPLE 0.5 mM FERULIC ACID TREATMENTS^a

Days of treatment	AGR			RGR		
	Growth periods (days)			Growth periods (days)		
	9-11	11-13	13-15	9-11	11-13	13-15
	51.89 ^a	73.82 ^a	97.50 ^a	1.27 ^a	0.70 ^a	0.52 ^a
7, 9	35.71 ^b	71.28 ^a	119.64 ^a	1.26 ^a	0.89 ^b	0.68 ^b
7, 9, 11	36.17 ^b	54.01 ^b	114.73 ^a	1.36 ^a	0.75 ^a	0.75 ^b
7, 9, 11, 13	32.23 ^b	45.93 ^b	61.26 ^b	1.27 ^a	0.70 ^a	0.51 ^a
LSD _{0.05}	8.66	16.51	31.39	0.23	0.14	0.12

^aColumn means followed by the same letters are not significantly different. AGR = cm²/2 days. RGR = cm²/cm²/2 days.

reduced by 67%, and root number, which more than doubled (Table 2) for the 0.5 mM ferulic acid treatments when compared to controls. If such changes in root morphology occurred in soil environments, acquisition of nutrients and water could be substantially impacted.

The suppression of seedling growth may be attributable to inhibition of photosynthesis and changes in source-sink relationships. Proportionally more of the radioactivity fixed by the photosynthetic process was retained by the shoots than was translocated to the roots. This is consistent with the observation that when growth is limited by shoot uptake of CO₂, shoot growth is relatively favored (Brouwer, 1983). The increasing root-shoot dry weight ratio observed in this study, however, does not support this hypothesis. The increase in the root-shoot ratio may be a result of differential lignification in shoots and roots of seedlings treated with ferulic acid (Shann and Blum, 1987b).

Transpiration and water utilization by cucumber seedlings was reduced by ferulic acid treatments (Blum and Dalton, 1985; Blum et al., 1985). In these studies it was noted that the reduction in water utilization was not due to the osmotic potential of the ferulic acid-nutrient solutions, because similar responses could not be generated by polyethylene glycol at equivalent milliosmoles. Changes in seedling turgor and osmotic pressure, closure of stomates, and reductions in mineral absorption have been noted for several other species when these were treated with ferulic acid (Einhellig et al., 1985; Balke, 1985). The rapid recovery of growth for both cucumber leaves and roots, once roots were removed from ferulic acid solutions, supports the hypothesis that root water uptake (not due to water potential of the solution) is a primary limiting factor and that effects on energy fixation may be secondary.

Finally, allelopathic constituents associated with organic matter in the soil are most likely unevenly distributed in soil, so that only a portion of a root system is impacted by such substances at any given time (Patrick et al., 1964).

Since the recovery of root growth was so rapid once ferulic acid was removed from the root environment, it is possible that the portion of the root system not in contact with allelopathic agents may grow normally and compensate for root inhibition experienced by roots in contact with allelopathic agents. The proportion of a root system required for contact with an allelopathic agent before growth inhibition of shoots can be detected remains undetermined.

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INVESTIGATION OF OVIPOSITION DETERRENT IN LARVAL FRASS OF *Spodoptera littoralis* (BOISD.)

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Abstract—Previous experiments demonstrated an oviposition-deterrent effect of larval frass in the Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.). In this study, females were shown to perceive the oviposition-deterrent substance(s) with their antennae. During dark, airtight, and cold (−10°C) storage, the deterrent was persistent for at least 395 days. On the other hand, larval frass retained its activity for only two days when applied to cotton leaves. The deterrent activity of frass was independent of larval density. Frass of larvae reared at high densities deterred oviposition as well as frass of larvae feeding separately or in small groups. For significant oviposition deterrence, the minimum amount of frass was in the range of 5–10 mg frass per cotton leaf. An acetone extract of larval frass was highly deterrent, in contrast to extracts prepared with water, ethanol, chloroform, or pentane.

Key Words—*Spodoptera littoralis*, Lepidoptera, Noctuidae, Egyptian cotton leaf worm, oviposition behavior, oviposition deterrence, larval frass.

INTRODUCTION

Host plants visited for oviposition by a phytophagous insect should offer sufficient food for the progeny. Since laying eggs on infested host plants might result in a lack of space and food for the hatching larvae, females may regulate ovi-

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position on a host plant according to the infestation density to obtain optimal developmental conditions for the hatching larvae. It has been shown for a wide array of phytophagous insect species that chemical messengers of either insect or plant origin inform females about the infestation of a host plant and thus regulate oviposition (Prokopy, 1981; Prokopy et al., 1984).

In several species of Lepidoptera, feeding larvae and larval frass respectively indicate occupancy of the host plant and deter egg deposition (e.g., Ditttrick et al., 1983; Mitchell and Heath, 1985; Renwick and Radke, 1980, 1981; Rothschild and Schoonhoven, 1977; Williams et al., 1986). Recently we found that oviposition is also deterred by larval frass in the Mediterranean noctuid moth *Spodoptera littoralis* (Boisd.) (Hilker, 1985).

To date, nothing was known about biological and chemical properties of this oviposition deterrent in *S. littoralis*. We hypothesized that only larvae at high densities excrete oviposition-detering substances to which females respond by avoiding egg deposition. Several studies of *S. littoralis* indicate a change of metabolism when larval density increases (e.g., Hodjat, 1970; Rivnay and Meisner, 1965; Zaher and Moussa, 1961). Metabolic changes might cause a change of frass compounds. These changed substances in the frass of larvae feeding at high densities might signal to gravid females when a site is unsuitable for oviposition. We therefore investigated whether the activity of larval frass is dependent on larval density. We have also studied the perception of the oviposition deterrent, its stability during cold storage, the persistence of the detering activity of larval frass on the host plant, and the solubility of the deterrent, and we have determined the minimum amount of frass that is necessary for significant oviposition deterrence. The results of these studies are reported here.

METHODS AND MATERIALS

For oviposition bioassays, moths of *S. littoralis* were obtained from a laboratory culture reared on semiartificial diet (Patana, 1969; Shorey and Hale, 1965). Only 3- to 4-day-old moths were used. Frass was collected daily from larvae of the fifth and sixth instar, feeding on cotton plant leaves. Only these late-instar larvae produced so much frass that sufficient material was available after a short time of collection. A determination of the fresh weight of frass production per day revealed that larvae feeding on cotton leaves excreted about 20–50 mg frass during the fifth instar and about 50–350 mg frass during the sixth instar, whereas third- and fourth-instar larvae produced only about 2–5 mg frass per day. Unless mentioned otherwise, the tested frass was 1–10 weeks old. After collection, frass was stored in dark, airtight conditions at -10°C . Cotton plants, *Gossypium barbadense* Mill., were grown in a greenhouse.

Bioassays were conducted in screened cages ($50 \times 50 \times 70$ cm) situated in a chamber with constant temperature ($27 \pm 1^\circ\text{C}$) and a 14 hr : 10 hr light-dark cycle according to conditions in Egyptian summer. Each bioassay began with the onset of the light period and lasted 24 hr. Three females and five males were placed in each cage. For oviposition, moths were offered two treated and two control cotton leaves. Each leaf was deposited in a 100 ml vial filled with water and situated in a corner of the cage.

With the exception of the bioassay testing the solubility of the deterrent, leaves were treated with a water suspension of frass. This suspension was prepared in a Potter homogenizer and applied to the undersurface of a leaf with a brush. Eggs are usually laid only on the undersurface of a cotton leaf. A test leaf was treated with 1 ml suspension and a control leaf with 1 ml water. Unless cited otherwise, the concentration of the suspension was 100 mg/ml H_2O . Six different experiments were conducted.

Experiment I. In order to determine the stability of the oviposition deterrent during storage, we tested larval frass stored in 15 ml vials at -10°C for either 6 or 395 days in airtight, dark conditions.

Experiment II. This experiment was conducted to determine the persistence of frass activity on the host plant. We bioassayed test and control leaves for 24 hr either immediately after treatment (persistence day 1), or during the second day after treatment (persistence day 2), or during the third day (persistence day 3). Prior to testing persistence day 2 and persistence day 3, leaves were kept in vials filled with water at room conditions.

Experiment III. In order to investigate the perception of the oviposition deterrent, antennae of female moths were removed. One hour before the antennae were ablated, the test females were put into a cold (-2°C) room. One hour later, the antennae of the motionless females could easily be cut at the base with small scissors. About 15 min after the moths had been taken out of the cold room, they showed normal activity. For control, females with intact antennae were used. The control females were also chilled at -2°C for 1 hr prior to testing.

Experiment IV. In order to determine the amount of frass that is necessary for significant oviposition deterrence, we tested suspensions in the following concentrations: 5 mg frass/ml H_2O , 10 mg frass/ml H_2O , and 100 mg frass/ml H_2O .

Experiment V. In order to analyze a possible influence of larval density on the activity of frass, we tested frass produced by larvae of the fifth and sixth instar kept at different densities. The following densities were calculated from the number of larvae per square cm area, where they could move: 0.002 larvae/cm² (one single larva per 500 cm²); 0.01 larvae/cm² (a small group of five larvae per 500 cm², moderate density); 0.07 larvae/cm² (35 larvae per 500 cm², overcrowded). All larvae were fed with cotton leaves in surplus.

Experiment VI. In order to get information on the solubility of the oviposition deterrent, we tried to extract it with different solvents. Extracts were prepared as follows: 1.1 g frass was added to 80 ml *n*-pentane, stirred for 12 hr at room temperature, and then filtered to obtain an *n*-pentane extract. The vacuum-dried residue was added to 80 ml chloroform, stirred for 12 hr, and newly filtered to obtain a chloroform extract. The extraction was continued in this way with acetone, ethanol, and water. The undersurface of each test leaf was treated with 1 ml of the various extracts using a soft brush. Each control leaf was treated with 1 ml of the respective solvent.

At the end of a bioassay, the number of egg masses on each leaf was counted. The number of replicates for the experiments was 23 with the exception of 20 in experiment VI. Statistical significance was tested by using the Wilcoxon test for paired differences.

RESULTS

The results of this study are compiled in Table 1.

Experiment I. Both larval frass stored for 6 days and for 395 days significantly deterred oviposition. Thus the oviposition deterrent was very stable when held in dark, airtight, and cold (-10°C) storage.

Experiment II. Larval frass distributed on cotton leaves deterred oviposition for two days. On the third day, cotton leaves treated with larval frass completely lost their oviposition-detering activity.

Experiment III. The oviposition deterrent was perceived with the antennae. Females without antennae were unable to differentiate between control leaves and leaves treated with larval frass. This was in obvious contrast to females with antennae.

Experiment IV. Frass at 5 mg per leaf did not significantly reduce oviposition, but a small effect was seen with 10 mg frass per leaf. On leaves treated with 10 mg frass, significantly fewer egg masses were deposited than on control leaves. The strongest oviposition deterrence was caused by 100 mg frass per leaf. The threshold value for statistical significance in oviposition deterrence was in the range of 5–10 mg frass per leaf.

Experiment V. The oviposition-detering effect of larval frass was independent of larval density. Frass of larvae feeding singly deterred oviposition as well as frass of larvae reared in moderate or high density.

Experiment VI. The oviposition deterrent was extractable from larval frass with acetone. The number of egg masses on cotton leaves treated with an acetone extract of frass was significantly lower than the number of egg masses on

TABLE 1. RESPONSE OF GRAVID *Spodoptera littoralis* FEMALES TO LARVAL FRASS AT DIFFERENT TEST CONDITIONS

Experiment	Test parameter	Total egg masses (%)			Significance ^a	Total No. egg masses (N)
		Test leaves	Control leaves			
I	Storage period					
	6 days ^b	25.0	75.0	*	40	
	395 days	22.2	77.8	**	36	
II	Persistence					
	1 day ^b	25.0	75.0	*	40	
	2 days	25.7	74.3	*	35	
	3 days	48.6	51.4	NS	37	
III	Perception					
	♀ ♀ with antennae	29.7	70.3	*	37	
	♀ ♀ without antennae	49.1	50.9	NS	55	
IV	Amount of Frass					
	5 mg/leaf	34.3	65.7	NS	35	
	10 mg/leaf	30.4	69.6	**	46	
	100 mg/leaf ^b	25.0	75.0	*	40	
V	Larval Density					
	0.002 larvae/cm ² (a single larva)	29.4	70.6	*	34	
	0.01 larvae/cm ² (a small group of larvae) ^b	25.0	75.0	*	40	
	0.07 larvae/cm ² (overcrowded)	31.1	68.9	*	61	
VI	Solubility					
	Pentane extract	35.7	64.3	NS	28	
	Chloroform extract	55.2	44.8	NS	29	
	Acetone extract	14.3	85.7	***	28	
	Ethanol extract	53.3	46.7	NS	30	
	Water extract	46.7	53.3	NS	30	

^aStatistically significant difference between number of egg masses on test and control leaves: * (0.05 ≥ P ≥ 0.01), ** (0.01 ≥ P ≥ 0.001), *** (P ≤ 0.001). NS, no significant difference.

^bOne single test listed several times for comparison. In this test we used larval frass stored under dark, airtight, and cold conditions for six days. Frass was obtained from larvae provided with cotton leaves and reared in small groups (0.01 larvae/cm²). Frass, 100 mg, was applied to each test leaf and bioassayed during the first day after application.

control leaves. All other tested extracts did not cause a significantly provable oviposition deterrence.

DISCUSSION

The results of this study provided information on the oviposition deterrent in *S. littoralis* and the first hints of its chemical nature. Moreover, useful clues resulted for further studies of the chemistry of the deterrent.

The investigation of the stability of the deterrent during cold storage demonstrated that there is no necessity of using fresh frass in order to show its oviposition-deterrent activity. During cold, dark, airtight storage the oviposition-deterrent was stable for longer than one year. Therefore, in chemical studies of old frass, stored at the above described conditions, it can be certain that active oviposition-deterrent substances are still present.

In contrast to the high stability of the oviposition deterrent during cold storage, its persistence was rather short when frass was applied to cotton leaves. Activity was lost after two days. Oviposition deterrents in other phytophagous insects showed a longer period of biological activity. The oviposition deterrents in larval frass of the corn borer, *Ostrinia nubilalis* Hb. (Dittrick et al., 1983), and the cabbage looper, *Trichoplusia ni* (Hb.) (Renwick and Radke, 1980), remained active for at least three days. The period during which the oviposition-deterrent pheromone of *Rhagoletis cerasi* L. retained its activity was at least 12 days (Katsoyannos, 1975). The oviposition-deterrent pheromone produced by females of *Pieris brassicae* L. was still active after it had been dried for seven weeks at room conditions in a desiccator (Schoonhoven et al., 1981).

In *S. littoralis* the development of all individuals of a population is not completely simultaneous. Therefore, it is possible that emergence of females coincides with the occurrence of feeding larvae. There should be no necessity for a long-lived oviposition deterrent, because feeding larvae continuously excrete fresh frass and, thereby, further oviposition-deterrent substances. The loss of activity of larval frass may be due to either evaporation or damage by oxygen or light.

Perception of the oviposition deterrent by the antennae does not provide evidence for olfactory perception. Gravid females often could be observed touching the leaves with their antennae. Therefore, perception by chemotactile sensilla should be considered. Helal and Abdel Gawaad (1984) investigated the antennae of *S. littoralis* males and females by means of scanning electron microscopy and found seven different types of sensilla. Electrophysiological

experiments are necessary in order to determine the sensilla responding to the oviposition deterrent in *S. littoralis*.

To our knowledge there is no other study that addresses sensory perception of oviposition deterrents when the deterrent is in larval frass. The oviposition-detering pheromone deposited by females of *Rhagoletis pomonella* Walsh is principally perceived by sensilla located on the tarsi (Crnjar et al., 1978; Prokopy and Spatcher, 1977). In addition to tarsal and probably abdominal contact chemoreceptors, in females of *Pieris brassicae* L. also olfactory sensilla located on the antennae show electrophysiological responses to the inherent oviposition-detering pheromone of the eggs (Behan and Schoonhoven, 1978; Klijnstra and Roessingh, 1986).

Females of *S. littoralis* need to perceive a minimum amount of larval frass before decreasing egg deposition. The threshold value for the quantity of frass causing a significant deterrence of egg laying was in the range of 5–10 mg frass per cotton leaf. Two days after moulting, a sixth-instar larva feeding on cotton leaves produces about 100 mg frass per day. About 10% of this daily frass production of a late-instar larva on a cotton leaf obviously indicated such a high feeding activity that a gravid female “decides” her offspring will not be sufficiently provided for at this site and therefore seeks another, more suitable, oviposition site. The result of this laboratory bioassay needs to be checked in the field in order to get information on ecological consequences of oviposition deterrence in *S. littoralis*. Field observations by Campion et al. (1977) revealed that *S. littoralis* emigrated from areas with high population densities. Possibly *S. littoralis* females respond to oviposition deterrents by emigration, in order to look for a place where the offspring will find suitable developmental conditions.

We previously hypothesized that frass activity is dependent on larval density. This hypothesis is based on the following background. Several studies demonstrated that an increase of larval density is correlated with numerous changes, e.g., larval color changes, activity of larvae increases, and fat and water content of the resulting pupae are different (e.g., Hodjat 1970; Rivnay and Meisner, 1965; Zaher and Moussa, 1961). These results indicate metabolic changes at higher larval density. Such metabolic changes may be correlated with changes of frass compounds. Possibly gravid females only avoid egg deposition in response to such changed frass compounds. These changed frass compounds would then indicate high larval density and, thus, unsuitable oviposition sites. Examination of this hypothesis revealed that the oviposition-detering activity of frass was independent of larval density. The oviposition-detering substances were obviously not subjected to metabolic changes when larval density increased.

The acetone solubility of the oviposition deterrent in frass of *S. littoralis* larvae indicates a moderate polar character of the deterring substances. The oviposition deterrent in larval frass of the European corn borer, *Ostrinia nubilalis* Hb., showed a similar solubility: methanol and acetone extracts of frass proved effective in reducing oviposition by 90% (Dittrick et al., 1983). A water extract of frass showed no activity in *S. littoralis*.

In contrast, the oviposition deterrent in larval frass of *Spodoptera exigua* L. and *Spodoptera eridania* (Cramer) could be extracted both with water and organic solvents like ethanol and dichloromethane (Mitchell and Heath, 1985). Oviposition in *Spodoptera frugiperda* (J.E. Smith) was also deterred by aqueous extracts of larval frass (Williams et al., 1986). The results of our study suggest that the oviposition-detering substances of *S. littoralis* will be chemically different from deterrents of other *Spodoptera* species. To date, the only identified oviposition deterrent is that released by females of *Rhagoletis cerasi* L.: Hurter et al. (1987) characterized this pheromone as *N*[15(β -glucopyranosyl)oxy-8-hydroxypalmitoyl]taurine.

Oviposition by *S. exigua*, *S. eridania*, and *S. frugiperda* was also deterred by extracts of damaged host-plant material (Mitchell and Heath, 1985; Williams et al., 1986). Furthermore, deposition of eggs by the noctuid moth *Trichoplusia ni* (Hb.) was reduced not only by larval frass, but also by damaged leaves of the host plants (Renwick and Radke, 1981). These results indicate that oviposition-detering substances in the larval frass of these species are undigested, allelochemical substances of the host plants. In *S. littoralis*, a suspension of macerated cotton leaves in water (100 mg/ml) did not deter oviposition (Hilker, 1985). This result does not prove that the oviposition deterrent in *S. littoralis* is a pheromone produced by the larvae themselves. In a suspension of macerated cotton leaves, oviposition-attracting substances might compete with oviposition-detering compounds that are possibly set free by damaging the leaves. In larval frass, the oviposition-attracting substances might be digested so far that undigested oviposition-detering plant substances would display their activity.

In our current work we hope to determine whether the oviposition deterrent in the larval frass of *S. littoralis* is a pheromone or an allelochemical plant substance.

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STRUCTURAL CORRELATION BETWEEN CUTICULAR
HYDROCARBONS AND FEMALE CONTACT SEX
PHEROMONE OF GERMAN COCKROACH
Blattella germanica (L.)

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Abstract—The structural relationships between the cuticular hydrocarbons and the contact sex pheromone of the female German cockroach, *Blattella germanica*, were investigated. Cuticular hexane extracts were separated into hydrocarbon and ketone fractions by TLC or silicic acid column chromatography. The ketone fraction (which contains the major contact sex pheromone component) was analyzed by GC-MS before and after reduction to hydrocarbon. In addition to 3,11-dimethyl-2-nonacosanone, 3,11-dimethyl-2-heptacosanone was also identified. Females have the 3,11- and 3,9-dimethyl C₂₇ and C₂₉ alkanes, but only the 3,11- isomer of the dimethylketones. In addition to the hydrocarbon components previously reported, a number of new components were characterized. Although the ratios of cuticular hydrocarbons differ among nymphs, adult males, and adult females, they have qualitatively identical hydrocarbon profiles, suggesting that the production of the contact sex pheromone results from the sex-specific oxidation of 3,11-dimethylalkanes to pheromone components by the female.

Key Words—Contact sex pheromone, cuticular hydrocarbons, 3,11-dimethyl-2-nonacosanone, 3,11-dimethyl-2-heptacosanone, German cockroach, *Blattella germanica*, Orthoptera, Blattellidae.

INTRODUCTION

The cuticular lipids of insects play a major role in protecting against desiccation and, in some cases, are involved in inter- and intraspecies chemical communication (Howard and Blomquist, 1982; Blomquist et al., 1987). Many components that function in chemical communication serve as short-range or contact pheromones because of their relatively high molecular weights and low vapor pressures.

The contact sex pheromone of the German cockroach, *Blattella germanica*, is present in cuticular extracts of adult females. This pheromone elicits in males a wing-raising courtship behavior that precedes copulation (Roth and Willis, 1952). The major component of the pheromone is 3,11-dimethyl-2-nonacosanone, with lesser amounts of 29-hydroxy-3,11-dimethyl-2-nonacosanone and 29-oxo-3,11-dimethyl-2-nonacosanone also reported (Nishida and Fukami, 1983). All three compounds independently stimulate males, resulting in a complete courtship response.

The major cuticular hydrocarbons of the German cockroach have been identified as homologous series of straight chain and methyl branched heptacosanes and nonacosanes (Augustynowicz et al., 1987). Since isomers of dimethylheptacosane and dimethylnonacosane were present in the cuticular hydrocarbons, we examined the methyl ketone pheromone fraction to determine if similar isomers and homologs were also present in this fraction. We report that in addition to 3,11-dimethyl-2-nonacosanone, 3,11-dimethyl-2-heptacosanone is also present. In addition to the hydrocarbon components previously reported (Augustynowicz et al., 1987), we report the structure of a number of other hydrocarbons on the surface of the German cockroach.

METHODS AND MATERIALS

Insects. Cockroaches were reared in 2-liter glass jars and fed Purina dog chow and water ad libitum. They were kept at 27°C with a 12:12 light-dark cycle. Males and females were separated on the day of adult emergence and 15-day-old insects were utilized.

Separation of Hydrocarbons and Pheromone. Insects were extracted by immersion in hexane for 10 min followed by two 1-ml hexane washes or were extracted by two 5-min hexane washes. The hexane extracts were combined and the solvent removed under a stream of nitrogen. Internal standards, hexacosane and 14-heptacosanone, were included during extraction to allow quantitation of hydrocarbons and ketones, respectively. Hydrocarbons and pheromone were separated by thin-layer chromatography (TLC) on silica gel type H, developed in hexane-diethyl ether (90:10 v/v) and the bands corresponding to hydrocarbons and the methyl ketone fraction scraped into test tubes

and extracted with diethyl ether. Alternatively, hexane extracts from individual insects were separated on Biosil A mini-columns (Howard et al., 1978).

Gas Chromatography and Gas Chromatography-Mass Spectrometry. Hydrocarbons and the ketone fraction were analyzed by gas chromatography (GC) with a flame ionization detector on both packed and capillary columns. Separations on packed columns utilized a 1.8-m \times 3.2-mm-ID glass column packed with Dexsil 300 on Supelcoport, temperature programmed from 200°C to 310°C at 5°C/min. Separations on capillary columns utilized a 60-m \times 0.32-mm-ID DB-1 column or a 15-m \times 0.53-mm-ID SPB-1 column temperature programmed from 80°C to 270°C at 20°C/min and then to 320°C at 3°C/min. Splitless injection was used on both capillary columns.

Gas chromatography-mass spectrometry (GC-MS) was performed on a Finnigan 4123 using an INCOS data system. Material was separated on a 30-m \times 0.72-mm capillary column temperature programmed from 200°C to 260°C at 2°C/min. A Finnigan 8200 high-resolution magnetic MS using a SS300 data system was also used. Material was separated on a 60-m \times 0.32-mm DB-1 capillary column with on-column injection.

Reduction of Methyl Ketone. The methyl ketone pheromone components were reduced by a modified Wolff-Kishner reaction (Huang-Minlon, 1946). The methyl ketone fraction (approx. 0.8–1.2 mg) was dissolved in 2 ml of diethylene glycol (DEG) to which 840 μ g NaOH in aqueous solution, 1.2 μ l hydrazine hydrate, and 1.0 mg 2-nonadecanone were added. The C-19 methyl ketone was added because preliminary studies indicated that the amount of pheromone was below the critical mass of the reaction conditions. The system was refluxed with a water-cooled condenser for 2 hr to allow formation of the hydrazone intermediate. The water was disconnected from the condenser and the reaction mixture gradually warmed to approximately 200°C, a temperature sufficient to evaporate the water and reflux DEG. After formation of crystalline material in the condenser, indicative of the presence of hydrocarbon, water was reconnected to the condenser, and the reaction refluxed for 4 hr more to decompose the hydrazone to hydrocarbon. The reaction mixture was then extracted three times with hexane and the reduced pheromone components purified on a 6-cm \times 0.5-mm Biosil A column by eluting with hexane. The hexane fraction was analyzed by GLC and found to contain the reduction products of the principal pheromone components. These were analyzed by GC-MS.

RESULTS

A chromatogram of the cuticular hydrocarbons from adult females separated by capillary GC is presented in Figure 1. The 15 principal hydrocarbon components have been identified previously by Augustynowicz et al. (1987) and are homologous series of heptacosanes and nonacosanes. We have now

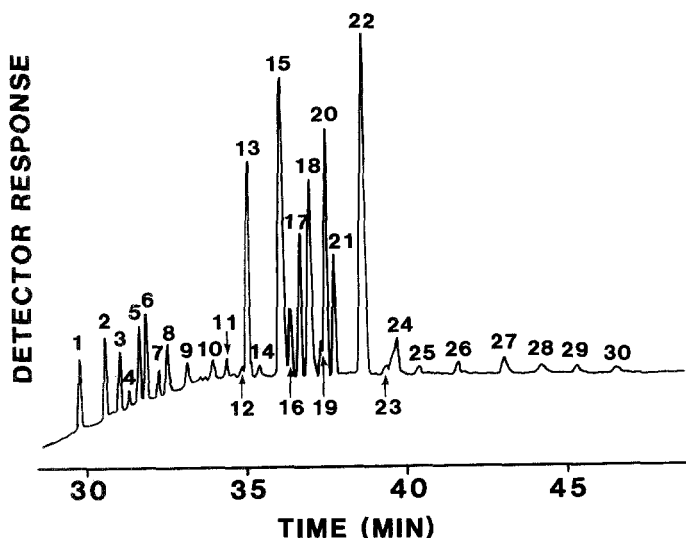


FIG. 1. A capillary column GC trace of the cuticular hydrocarbons from 15-day-old virgin German cockroach females. Peaks are numbered according to the components identified in Table 1.

identified a number of minor components, additional isomers of the major components, and the unknown dimethylalkanes not determined previously (Augustynowicz et al., 1987).

The previously unidentified dimethylheptacosane (peak 4, Figure 1) has the methyl groups in the 11,15- positions with diagnostic ion fragments at m/z 168/169, 196/197, 239, and 267. The fragments containing a single methyl branch have an odd-to-even ratio of about 1 and the fragments that contain a second methyl branch have a higher ratio of odd-to-even mass units. In each spectrum referred to here, if the odd-to-even ratio were near 1, both fragments are listed, whereas if the odd-numbered fragments clearly dominate, only one mass number is listed. The previously unidentified dimethylnonacosane (peak 21, Figure 1) is a mixture of 5,9- and 5,11- isomers with diagnostic ion fragments at m/z 84/85, 155, 308/309, 183, 280/281, and 379. In addition to 13,17-dimethylnonacosane, this GC peak (peak 18, Figure 1) also has an isomer with methyl groups in the 11,15- positions (m/z 168/169, 224/225, 239, and 295). We have also identified additional isomers in the dimethylheptacosane series. In addition to 5,11-dimethylheptacosane, the 5,9- isomer is also present [m/z 351 ($M-57$)⁺, 155, 280/281] (peak 6, Figure 1) and in addition to 3,11-dimethylheptacosane, the 3,9- isomer is also present [m/z 379 ($M-29$)⁺, 155, 280/

281] (peak 8, Figure 1). In addition to the 11-, 13-, and 15-methylnonacosane the 9- isomer is also present (m/z 140/141 and 308/309) (peak 15, Figure 1).

Minor components not identified in the paper by Augustynowicz et al. (1987) consist of 2-methyloctacosane [m/z 365 ($M-43$)⁺] (peak 10, Figure 1) and 4-methyloctacosane [m/z 365 ($M-43$)⁺, 337/336 ($M-71/72$)⁺] (peak 11, Figure 1). Both 2- and 4-methylalkanes give strong ion fragments at m/z ($M-43$)⁺, whereas 4-methylalkanes also show ion fragments at ($M-71$)⁺ and ($M-72$)⁺ (Blomquist et al., 1987). Other minor components present are 4,8- and 4,10-dimethyltriacontane [m/z 407 ($M-43$)⁺, 141, 169, 308/309 and 336/337], 11-, 13-, and 15-methylhentriacontane (m/z 168/169, 206/207, 224/225, 252/253, 280/281 and 308/309), 13,17- and 11,15-dimethylhentriacontane (m/z 168/169, 196/197, 224/225, 239, 267, 252/253, 295 and 323), 5,9- and 5,11-dimethylhentriacontane [m/z 407 ($M-57$)⁺, 84/85, 155, 183, 308/309 and 336/337], and 10- and 12-methyldotriacontane (m/z 154/155, 182/183, 308/309 and 336/337).

The percent composition of the hydrocarbon fraction from males, females, and nymphs is presented in Table 1. No qualitative differences were observed between the different life stages or sexes, and the 3,*x*-dimethylalkanes were present in all groups. However, consistent quantitative differences were observed between 15-day-old adult males and virgin females (Table 1). The most prominent components extracted from cuticles of females were isomers of 3,*x*-dimethylnonacosane ($18.6 \pm 0.7\%$, $32.4 \pm 1.4 \mu\text{g}$), while isomers of internally branched monomethylnonacosanes (9-, 11-, 13-, and 15-) were the most abundant components of males ($22.7 \pm 0.6\%$, $41.0 \pm 2.2 \mu\text{g}$). Last nymphal instar males also have more 9-, 11-, 13-, and 15-methylnonacosanes than last instar females.

A chromatogram of the methylketone fraction after isolation by TLC and analysis by packed column GC is presented in Figure 2. The major components are 3,*x*-dimethyl-2-nonacosanone (79.2%) and 3,*x*-dimethyl-2-heptacosanone (8.1%). Since the cuticular hydrocarbons had isomers of dimethylnonacosane in the 3,11-, 3,9-, and 3,7- positions and dimethylheptacosane in the 3,11- and 3,9- positions, we determined whether these same positional isomers are present in the methyl ketone pheromone fraction. Mass spectra of dimethyl-2-nonacosanone and dimethyl-2-heptacosanone are shown in Figure 3 with the M⁺ ion fragments at m/z 450 and 422, respectively. The ion fragments at m/z 127, 141, 155, 169, and 197 are indicative of a 2-keto group with an adjacent methyl branch in the hydrocarbon chain. The position of the methyl groups could not be determined from these data.

The methyl branch positions of dimethyl-2-nonacosanone and dimethyl-2-heptacosanone were determined by reducing the ketone to the hydrocarbon followed by GC-MS analysis. The mass spectra are presented in Figure 4. The

TABLE 1. PERCENT COMPOSITIONS OF CUTICULAR HYDROCARBONS FROM LAST INSTAR NYMPHS AND ADULT MALE AND FEMALE *Blattella germanica*

	Percent composition			
	Adult		Last instar	
	Male	Female	Male	Female
1. <i>n</i> -Heptacosane	1.8 ± 0.2	2.1 ± 0.3	1.7 ± 0.2	3.7 ± 0.4
2. 11- and 13-Methylheptacosane	4.6 ± 0.3	2.6 ± 0.3	3.1 ± 0.3	4.4 ± 0.3
3. 5-Methylheptacosane	2.5 ± 0.2	1.8 ± 0.1	3.4 ± 0.3	3.9 ± 0.3
4. 11,15-Dimethylheptacosane	0.6 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	1.0 ± 0.2
5. 3-Methylheptacosane	2.9 ± 0.1	2.7 ± 0.2	3.2 ± 0.5	3.7 ± 0.3
6. 5,9- and 5,11-Dimethylheptacosane	1.4 ± 0.3	1.7 ± 0.2	3.7 ± 0.4	4.3 ± 0.1
7. <i>n</i> -Octacosane	0.8 ± 0.1	1.4 ± 0.2	1.3 ± 0.4	1.5 ± 0.4
8. 3,11- and 3,9-Dimethylheptacosane	1.4 ± 0.3	1.9 ± 0.2	4.9 ± 0.5	5.4 ± 0.2
9. 12- and 14-Methyloctacosane	2.0 ± 0.2	2.0 ± 0.3	2.7 ± 0.7	1.9 ± 0.4
10. 2-Methyloctacosane	1.2 ± 0.2	1.3 ± 0.2	1.6 ± 0.5	1.5 ± 0.3
11. 4-Methyloctacosane	1.2 ± 0.2	1.0 ± 0.2	1.5 ± 0.7	1.5 ± 0.5
12. Unknown	0.5 ± 0.1	0.4 ± 0.1	1.1 ± 0.4	1.1 ± 0.2
13. <i>n</i> -Nonacosane	6.0 ± 0.3	7.4 ± 0.2	5.8 ± 0.5	8.0 ± 0.6
14. Unknown	1.0 ± 0.2	0.5 ± 0.1	1.5 ± 0.5	1.2 ± 0.5
15. 9-, 11-, 13-, and 15-Methylnonacosane	22.7 ± 0.6	14.5 ± 0.2	16.3 ± 3.3	11.2 ± 0.3
16. 7-Methylnonacosane	3.3 ± 0.1	2.5 ± 0.1	2.9 ± 0.3	3.0 ± 0.1
17. 5-Methylnonacosane	6.2 ± 0.2	5.5 ± 0.2	6.8 ± 0.5	6.4 ± 0.4
18. 13,17- and 11,15-Dimethylnonacosane	5.9 ± 0.2	8.6 ± 0.5	3.1 ± 0.4	3.8 ± 0.2
19. Unknown	0.9 ± 0.04	1.0 ± 0.1	0.8 ± 0.2	1.2 ± 0.1
20. 3-Methylnonacosane	8.6 ± 0.5	10.3 ± 0.4	6.0 ± 0.6	6.3 ± 0.2
21. 5,9- and 5,11-Dimethylnonacosane	4.8 ± 0.2	5.8 ± 0.3	4.0 ± 0.6	4.5 ± 0.2
22. 3,7-, 3,9-, and 3,11-Dimethylnonacosane	14.0 ± 0.4	18.6 ± 0.7	19.6 ± 2.5	16.1 ± 1.3
23. Unknown	0.9 ± 0.1	0.5 ± 0.02	0.8 ± 0.3	0.6 ± 0.1
24. 11-, 13-, and 15-Methyltriacontane	1.1 ± 0.1	2.2 ± 0.1	1.4 ± 0.2	1.7 ± 0.2
25. Unknown	0.2 ± 0.02	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
26. 4,8- and 4,10-Dimethyltriacontane	0.5 ± 0.03	0.8 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
27. 11-, 13-, and 15-Dimethylhentriacontane	2.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
28. 13,17- and 11,15-Dimethylhentriacontane	0.4 ± 0.04	0.6 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
29. 5,9- and 5,11-Dimethylhentriacontane	0.4 ± 0.1	0.5 ± 0.03	0.2 ± 0.1	0.4 ± 0.1
30. 10,12-Dimethyldotriacontane	0.2 ± 0.04	0.1 ± 0.05	0.0	0.2 ± 0.1
Total (μg/insect)	180.6 ± 7.0	174.2 ± 5.6	33.8 ± 2.8	37.3 ± 0.7

ion fragments at 183, 252/253, and 379 in Figure 4A indicate that only the 3,11- positional isomer of dimethylheptacosane is present. The same is true for the 31-carbon component with the ion fragments at 183, 280/281, and 407, indicating that only the 3,11- isomer is present (Figure 4B). These data indicate

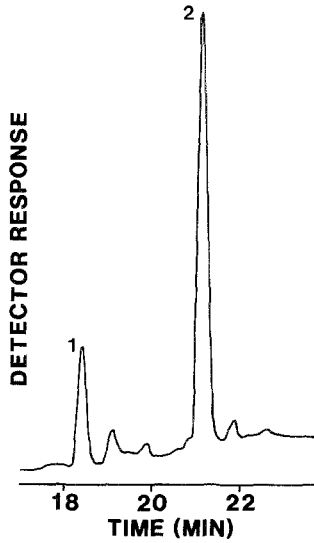


FIG. 2. A packed column GC trace of the methyl ketone fraction from 15-day-old virgin German cockroach females. 1 = 3,11-dimethyl-2-heptacosanone, 2 = 3,11-dimethyl-2-nonacosanone.

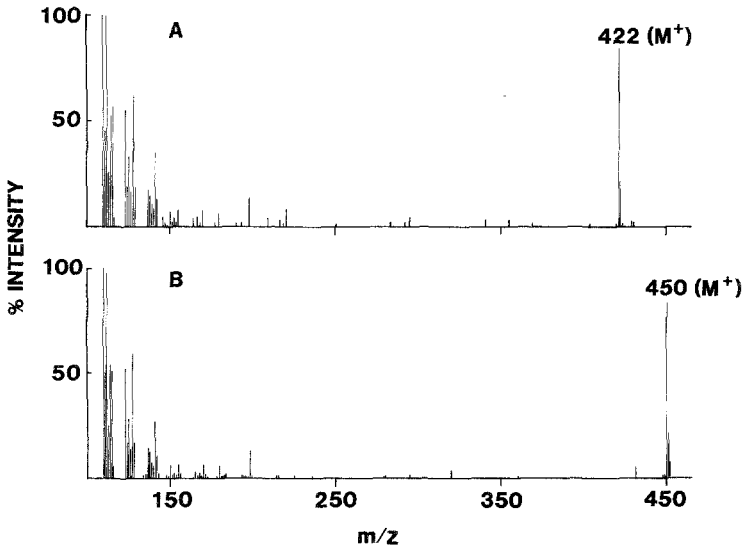


FIG. 3. Mass spectra from the methyl ketone fraction: (A) 3,11-dimethyl-2-heptacosanone; (B) 3,11-dimethyl-2-nonacosanone.

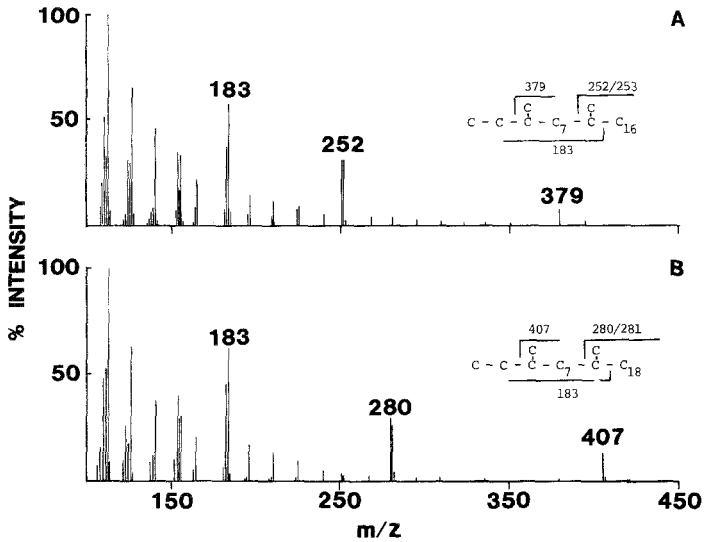


FIG. 4. Mass spectra of the reduced methyl ketone fraction from female German cockroaches: (A) reduced 3,11-dimethyl-2-heptacosanone; (B) reduced 3,11-dimethyl-2-nonacosanone.

that the principal dimethylketone component is 3,11-dimethyl-2-nonacosanone with lesser amounts of 3,11-dimethyl-2-heptacosanone also present.

DISCUSSION

The principal cuticular hydrocarbons of the German cockroach are composed of homologous series of straight chain, mono-, and dimethyl heptacosanes and nonacosanes (Augustynowicz et al., 1987). Also present as minor components are homologous series of hentriacontane except that components with the 3,*x*- branching patterns are absent.

In female German cockroaches, the 3,*x*-dimethylalkanes comprise the major type of hydrocarbon. Hydrocarbons with 3,*x*-dimethyl branching patterns were first reported in the fire ants, *Solenopsis invicta* and *S. richteri* (Nelson et al., 1980), where they were present in trace amounts. This type of hydrocarbon has also been reported in the housefly, *Musca domestica* (Nelson et al., 1981) and more recently in several other species (Blomquist et al., 1987). However, in all the previous species examined that contain 3,*x*-dimethylalkanes, they are present in trace amounts. The relatively large amounts of the 3,*x*-dimethylalkanes in the female German cockroach may reflect their use as the biosynthetic precursor to the dimethylketone contact pheromone components.

The 3,*x*-dimethylheptacosanes and nonacosanes both contain 3,11- and 3,9-dimethyl isomers with the nonacosane series also containing the 3,7- isomer. The methylketone component of the pheromone has been identified as only one isomer, the 3,11-dimethyl-2-nonacosanone (Nishida et al., 1974). Since the cuticular hydrocarbons contain at least two positional isomers of 3,*x*-dimethylnonacosane and dimethylheptacosane, we performed experiments to determine if female cockroaches also had the same homologs and positional isomers of methyl ketones as they have for the hydrocarbons. Unlike the hydrocarbons, the C₃₁ methyl ketone component of the pheromone contains only the 3,11-isomer, as was also shown by Nishida et al. (1974). We have found, however, that the heptacosanone homolog is also present, but again, only as the 3,11-isomer.

The structural similarities between the cuticular hydrocarbons and the contact pheromone suggests a similar biosynthetic origin. Malonyl-CoA serves as the two-carbon donor for chain elongation during hydrocarbon synthesis. In insects methyl branches in cuticular hydrocarbons arise from the substitution of methylmalonyl-CoA for malonyl-CoA at specific biosynthetic steps during this elongation (Blomquist et al., 1987). Various substrates have been shown to serve as precursors to methylmalonyl-CoA. In the housefly, *Musca domestica*, studies with [1-¹³C] propionate showed that 3,*x*-dimethylalkanes were formed by the insertion of propionates, as methylmalonyl-CoA derivatives, during the early stages of chain elongation (Dillwith et al., 1982). The source of the propionyl-CoA and methylmalonyl-CoA used in branched alkane biosynthesis has been shown to be the amino acids valine and isoleucine in the housefly and American cockroach (Dillwith et al., 1982; Halarnkar et al., 1985). In termites, succinate is converted to the methylmalonyl unit that forms the methyl branch group (Chu et al., 1980; Blomquist et al., 1980).

Two possible explanations exist to account for the presence of the carbonyl group at the 2-position of the pheromone component. The female cockroach could oxidize the preformed hydrocarbon at this position or fail to reduce the carbonyl group that is present at this position during the condensation reaction by which the hydrocarbon is synthesized. The epoxides in the sex pheromone of the housefly (Blomquist et al., 1984) and the gypsy moth (Prestwich, 1987; Kasang, 1974) arise from the insertion of an oxygen into a preformed alkene. Similarly, the carbonyl group in the (*Z*)-14-tricosene-10-one pheromone component of the female housefly arises from the insertion of an oxygen into a preformed chain. A cytochrome P-450 polysubstrate mixed function oxidase hydroxylates the carbon chain, and the secondary alcohol is then apparently oxidized to the ketone (Ahmad et al., 1987).

Assuming that the methyl ketone pheromone components arise from a preformed hydrocarbon, the enzyme(s) involved in synthesis of the pheromone would require a high degree of specificity for a 3,11-dimethylalkane branching

pattern but apparently are not as specific as to chain length. Only the 3,11-isomers were found in the methyl ketone fraction, but both heptacosanone and nonacosanone homologs were present. The absence of 3,11-dimethylhentria-cosane in the hydrocarbon profile and absence of the corresponding methyl ketone also suggests specificity of the enzyme for a 3,11- isomer. Indeed, the ratio of 3,11-dimethylheptacosanone to its hydrocarbon analog (4.4) is similar to the ratio of 3,11-dimethyl-2-nonacosanone to its hydrocarbon analog (4.3), suggesting equal efficiency in conversion of both hydrocarbons to their respective methyl ketones.

It appears that only adult females have the enzymes necessary to synthesize the methyl ketone pheromone. Both 3,11-dimethyl C₂₇ and C₂₉ hydrocarbons are present in males, but apparently the enzyme system is not. We report different hydrocarbon composition profiles from that previously reported (Augustynowicz et al., 1987). Augustynowicz et al. (1987) found that the 11-, 13-, and 15-methylnonacosane isomers were most abundant (16.5%), followed by 3- (14.7%) and 5-methylnonacosane (10.4%). The 3,7-, 3,9-, and 3,11-dimethylnonacosane isomers made up 11.3% of the cuticular hydrocarbons. We report that in adult females the 3,7-, 3,9-, and 3,11-dimethylnonacosane isomers make up the largest group by percent composition (18.6%) followed by the 9-, 11-, 13-, and 15-methylnonacosane isomers (14.5%). In adult males the most abundant hydrocarbons were the 9-, 11-, 13-, and 15-methylnonacosanes (22.7%), whereas the 3,*x*-dimethylnonacosane isomers make up only 14.0% of the hydrocarbon fraction (Table 1). These differences between our results and those of Augustynowicz et al. (1987) are clearly due to the combining of male and female cockroaches in their analysis.

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PRIOR CROPPING WITH GRAIN SORGHUM INHIBITS WEEDS

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Abstract—Three years of field data in northeastern Nebraska demonstrate that a grain sorghum crop reduces weediness in the following crop year. Weed growth was consistently lower in sorghum areas the year after strip-cropping fields with sequences of four-row bands of grain sorghum, soybeans, and corn. Percentage weed cover was significantly lower early in the year, and midsummer weed biomass was well below that found after corn and soybeans. Weed biomass in June and July following corn was two to four times that of grain sorghum strips. Inhibitory effects of grain sorghum were primarily on broadleaf weeds, often showing no action on grass weeds. No obvious differences were noted in the weed species present after the three crops. Allelopathy provides a logical explanation for the sorghum-mediated weed inhibition found in this study. The data have implications for weed management strategies in agriculture.

Key Words—Allelopathy, grain sorghum, *Sorghum bicolor*, weed inhibition, weed management.

INTRODUCTION

Cultivated *Sorghum* species have a history of use in weed management. Overland (1966) noted that they were among those crops used as "smother crops," with the implication that they competitively suppressed weed populations growing during the same time period. Recently, Putnam et al. (1983) showed that grain sorghum [*Sorghum bicolor* (L.) Moench] and sudangrass [*Sorghum sudanense* (Piper) Stapf.] were useful cover crops for controlling weeds in orchards.

Residues from frost-killed sorghum planted in the intertree space in apple and cherry orchards inhibited weed growth. A sorghum \times sudangrass hybrid used in these studies reduced the weed biomass to less than 40% of that found without a cover crop. In annual cropping systems, planting without tillage into a desiccated cover crop of sorghum or sudangrass almost totally eliminated certain weed problems (Putnam and DeFrank, 1983). At least part of these effects were attributed to allelopathy.

Sorghum species contain a variety of water-soluble substances that can inhibit seed germination and seedling growth. Guenzi and McCalla (1966a) isolated substantial quantities of ferulic, *p*-coumaric, vanillic, syringic, and *p*-hydroxybenzoic acids from decomposing *S. bicolor* residues. Subsequent work indicated phytotoxicity from these compounds could persist in field conditions for at least 28 weeks (Guenzi and McCalla, 1966b; Guenzi et al., 1967). Lehle and Putnam (1983) found inhibitory activity from several chemical fractions separated after aqueous extraction of herbage from *S. bicolor* cv. Bird-a-boo, and these fractions included more than phenolic acids. Phytotoxicity from germinating seeds, root exudates, and aqueous extracts of foliage has also been reported from other *S. bicolor* cultivars (Hussain and Gadoon, 1981; Panasiuk et al., 1986). Extracts from various organs of *S. vulgare* Pers., a forage crop, and Johnson grass, [*S. halepense* (L.) Pers.], a troublesome weed, contain allelopathic chemicals (Alsaadawi et al., 1986; Abdul-Wahab and Rice, 1967). The latter produces dhurrin and taxiphyllin, two cyanogenic compounds which yield HCN and *p*-hydroxybenzaldehyde upon hydrolysis (Nicollier et al., 1983). Apparently a variety of compounds may contribute to any suspected cases of *Sorghum* allelopathy.

Agricultural weed control alternatives to the present commercial herbicide-dominated programs are now being given wide consideration (Einhellig and Leather, 1988). As noted, there is compelling evidence that planting into a sorghum cover crop residue may provide weed control. Weed suppression has also been obtained when fall-seeded alfalfa immediately follows a summer *Sorghum* forage (Forney et al., 1985). What is less well established is the year-to-year carryover weed-control capability of a sorghum crop. Hence, these investigations were undertaken to determine the impact of *S. bicolor* on the weed population in a subsequent year. Their initiation was stimulated from observations in a working farm situation.

METHODS AND MATERIALS

Field Site. The study sites were located on a farm in northeastern Nebraska, known locally as the Gary Young farm (South Half of 3129, One West, Cedar County, Nebraska). Soils of this area are Peoria loess and are deep, well-drained, and eroded (Milliron, 1985). The history of the fields was well docu-

mented, and there was no record of herbicide or commercial fertilizer application. In the study years, crops were planted using a no-till approach, and no herbicide, pesticide, or fertilizer were employed. Data from the first year (1985) were from a field with Nora silty clay loam with 6–11% slope. The other two years' data were collected from two fields with Moody silty clay loam soil, 2–6% slope. Both soil types are closely related and are described as being suited for dry land corn, soybeans, oats, and alfalfa. Our soil analysis showed 29% clay with a pH of 7.1 for the first year field, and 27% clay, pH 6.9, for the latter years. These analyses are in agreement with the county soil survey (Milliron, 1985).

The data collection plan for the first year was to analyze weed conditions the year after fields had been strip cropped with alternating, four-row bands of grain sorghum [*Sorghum bicolor* (L.) Moench] and soybeans [*Glycine max* (L.) Merr.]. In the next two years, corn (*Zea mays* L.) was added as a third crop in the strip-cropping sequences. Crops were planted on a 38-in.-row spacing, and the cultivars of the crops planted were different each year. Early weed cover the following year was obtained from sampling the entire field. Biomass sampling of weeds later in the season was obtained from within adjacent 12-m lengths of the former strip crops, which were left unplanted during the sampling year.

Data Collection. The point-contact method was used to quantify weed cover in May of the year after the crops (Crockett, 1964). Sampling was conducted using a point frame with 10 contact points spaced 10 cm apart. Each previous crop area was systematically sampled with 50 point frames, making a total of 500 points. Care was taken to avoid sampling the edges of the strip plots. Points hitting aerial cover of grass and broadleaf weeds were recorded. The data were analyzed by considering each point frame as a sample. Differences between the treatments in grass weeds, broadleaf weeds, and total weed cover were ascertained using analysis of variance (ANOVA) with Duncan's multiple-range test.

Later in the growing season (June and July), systematic sampling of above-ground weed biomass involved clear-clipping five 0.5-m² quadrats in each of the undisturbed prior-year crop areas. Weeds were separated into grass weeds and broadleaf weeds, and dry weights were obtained after 48 hr at 105°C. Statistical comparisons of the grass, broadleaf, and total weed biomass components between crop areas were made using ANOVA as previously described.

RESULTS

Data from all three years of this study demonstrated that the nature of the crop species in one year influenced weed growth in the subsequent year. Weed reduction in areas where grain sorghum had been the previous year was often

apparent from visual inspection. During the first year of data collection, 1985, the weed population was monitored in a field which had been cropped in 1984 with alternating bands of grain sorghum and soybeans. When the field was viewed in early May 1985, strips where the soybeans had been the previous year were quite green, compared to a more barren appearance in the grain sorghum strips.

Point-frame sampling data obtained on May 7 established that grain sorghum strips had about one fourth the weed cover found in the soybean rows (Figure 1). Both grass and broadleaf weeds were significantly less where sorghum had grown the year before. By May 28, the aerial weed cover in the sorghum strips was 80% of that found after soybeans, a differential that was not statistically different. However, ANOVA showed that the broadleaf component of this weed cover was still significantly lower in grain sorghum. Visual observations at this time also suggested that the weed biomass was much less in sorghum strips.

Weed biomass in quadrats sampled on June 18 averaged 27 g for prior sorghum areas, compared to 48 g in soybeans (Figure 1). The biomass of broadleaf weeds in soybean areas was two and one-half times that of sorghum,

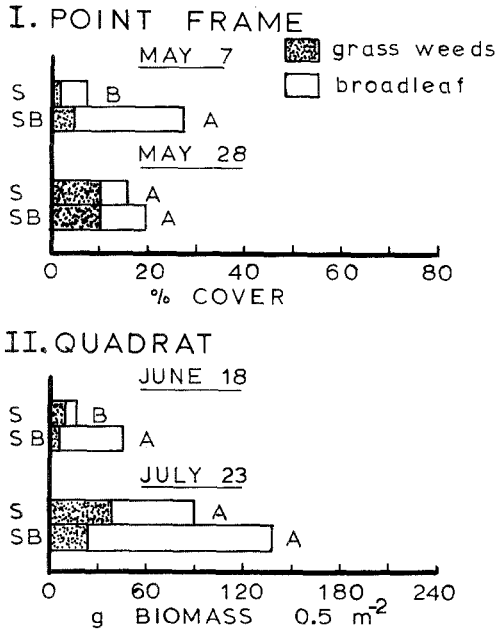


FIG. 1. Effects of the prior year (1984) crop on weed abundance in 1985. S = grain sorghum; SB = soybeans. Bars within each sampling date having different letters are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

whereas grass weeds were significantly higher in the sorghum. Sampling in July still showed grain sorghum strips had less weed biomass, but there was much more variability among quadrats, and the difference in total biomass between the treatments was not significant. *Setaria* spp. appeared to make up a greater component of the weeds found in sorghum strips. Other species noted in both areas included *Polygonum pensylvanicum* L., *Kochia scoparia* (L.) Schrad., *Lactuca serriola* L., *Solanum rostratum* Dun., *Ambrosia trifida* L., *Amaranthus retroflexus* L., *Convolvulus arvensis* L. and *Physalis subglabrata* Mackenz. & Bush.

Fields in the second and third year had corn added to the strip cropping so that comparisons might be made with a crop where there would be no question about effects from residual nitrogen fertility. At each of the sampling dates in both 1986 and 1987, the weed growth was significantly lower in strips of the prior year sorghum than either soybeans or corn (Figures 2 and 3).

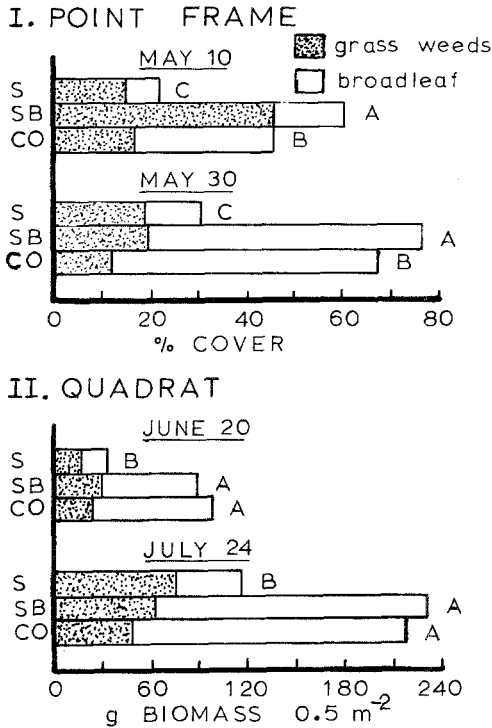


FIG. 2. Effects of the prior year (1985) crop on weed abundance in 1986. S = grain sorghum; SB = soybeans; CO = corn. Bars within each sampling date having different letters are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

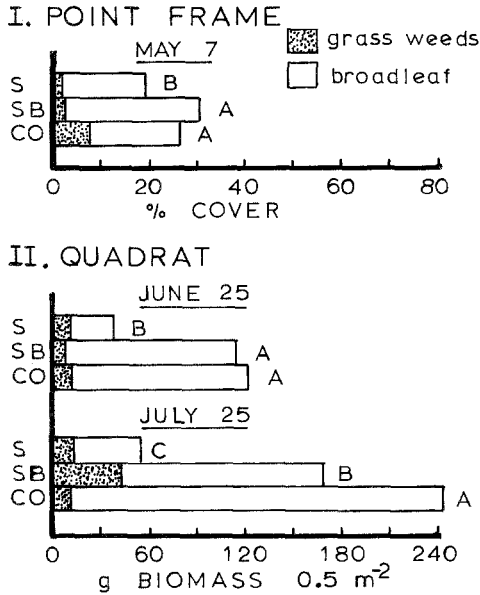


FIG. 3. Effects of the prior year (1986) crop on weed abundance in 1987. S = grain sorghum; SB = soybeans; CO = corn. Bars within each sampling date having different letters are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

Weed cover from all weeds in grain sorghum areas early in the 1986 season was approximately one third of that found after soybeans, and one half that following corn (Figure 2). This was also the pattern seen for the differences in broadleaf weeds among the three treatments, whereas grasses were not significantly different between sorghum and corn. At the second sample, weed cover in grain sorghum was 40 and 45%, respectively, of that in the prior year soybean and corn. These differences reflected the significant reduction in broadleaf weed cover following grain sorghum. In contrast, grass weed cover in corn was slightly less than in the other two prior crops. Data from both June and July showed a significant reduction in weed biomass after grain sorghum (Figure 2). The total biomass in grain sorghum plots on June 20 was approximately one third of that found after corn, and on July 24 these same areas were 54% of corn plots. Essentially all of these differences were in the broadleaf component of the biomass. No marked differences in the species composition among the three treatments were observed.

In the third year, treatment effects on weed abundance early in the season could not be as readily seen as they had been in the other two years. However, data obtained on May 7 showed aerial weed cover was significantly lower in the prior year grain sorghum areas than either of the other two crops (Figure

3). Favorable temperatures resulted in very rapid early weed growth in 1987, so a second assessment of cover was not obtained. In June, it was easy to see the contrast between grain sorghum and the other two treatments. Although grain sorghum plots had less weed growth, the distribution of species appeared similar across the three areas, and these were similar to previous years. Grain sorghum quadrats on June 25 averaged less than one third the weed biomass found in plots of the prior year corn or soybean (Figure 3). All of the biomass difference was from a suppression of broadleaf weeds. The July corn quadrats had more than four times the biomass of the grain sorghum quadrats, and at this sampling corn areas supported more weed biomass than the soybean areas.

DISCUSSION

The data show weed abundance in the year following grain sorghum was markedly suppressed in comparison to the weed conditions after either corn or soybeans. These results were obtained in the absence of tillage. The impact of grain sorghum was not an absence of weeds, but one of delayed emergence and growth inhibition. Total biomass accumulation late in the season was well below that which occurred in the nonsorghum areas. The reproducibility of these results is evidenced by the fact that they occurred in three different fields and with variations in moisture (Table 1) and other climatic conditions over the three year study.

Edaphic factors do not provide a logical explanation for the reduction in

TABLE 1. MONTHLY PRECIPITATION (cm) BETWEEN CROP PLANTINGS AND SUBSEQUENT YEAR SAMPLING PERIOD

Month	1984-1985	1985-1986	1986-1987
June	21.3	11.9	14.9
July	8.5	3.8	3.1
August	2.0	16.0	7.0
September	2.1	9.1	11.2
October	10.5	2.7	5.9
November	2.8	2.8	1.3
December	2.2	0.0	0.2
January	0.7	0.3	0.3
February	0.0	0.0	1.4
March	0.4	0.3	19.2
April	17.0	5.3	2.6
May	11.1	7.5	11.7
June	11.9	14.9	13.0
July	3.8	3.1	11.5
14-Month total	94.3	80.7	103.3

weeds the year after grain sorghum, since the test areas were side-by-side strips having the same soil type and receiving a comparable quantity of moisture. It is possible that weed germination in soybean areas could have been stimulated by nitrates that might be higher in these areas (Roberts and Smith, 1977; Vincent and Roberts, 1977). This might account for the greater weed cover in soybeans than corn plots in 1986. It cannot explain the fact that weed cover in the corn was still more than twice that found following grain sorghum. If there was any bias of the physical conditions between the corn and grain sorghum areas, it was that the former had a more extensive surface cover of crop residue than occurred with grain sorghum, and this might be expected to slightly delay soil warming in the corn plots. In spite of this, early emergence and subsequent growth of weeds was greater where corn had been.

Allelopathic conditions from grain sorghum as the donor plant must be considered as a major factor in the weed inhibition. We suspect that no one compound mediates this allelopathy, but that it is the result of the collective action of several compounds (Einhellig et al., 1982; Lehle and Putnam, 1983; Einhellig, 1987). The focus of this research was not on isolation and identification of the allelochemicals, but it is likely they included cyanogenic glycosides and a variety of phenolic acids and aldehydes that have been previously reported (Guenzi and McCalla, 1966a; Martin et al., 1938).

The extent of any sorghum-mediated allelopathy will be influenced by environmental conditions, both with regard to production of allelochemicals and seasonal carryover. The level of accumulation of many phenolic and coumarin allelochemicals in a plant is influenced by plant age, light intensity, and numerous stress conditions (Woodhead, 1981; Lehle and Putnam, 1982). Rainfall could be important to the persistence of allelochemicals into the next crop year. However, the data over the three years showed similar effects in all years even though the second year was drier than the others. It is possible that the heavy rainfall in March of the third year may have reduced the degree of difference in weed cover among the plots in May of 1987.

Recognition that under no-till conditions *S. bicolor* can suppress weeds in the year following the crop has potential applications in agriculture. Any such application strategies should be tested with intended tillage operations, crop cultivars, rotation sequences, and climatic and soil conditions of the region. It has also been shown that some allelochemical inhibitions can work in conjunction with commercial herbicides (Einhellig, 1987). Hence, grain sorghum might be used in a crop rotation sequence either with or in the absence of other weed control measures. In any case, the planned use of a grain sorghum crop as a provision for weed management could reduce reliance on herbicides.

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MONOTERPENOID CONTENT OF SAGE GROUSE INGESTA¹

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Abstract—We tested the hypothesis that the monoterpenoid levels in the ingesta from various digestive organs of sage grouse are less than that expected from the big sagebrush leaves ingested. Results supported the hypothesis. Dramatic reductions occurred between the gizzard and duodenum. Monoterpenoid levels in the ceca were nil; thus adverse effects of monoterpenoids on ceca microbes would also be nil.

Key Words—volatile oils, essential oils, digestion, *Centrocerus urophasianus*, big sagebrush, *Artemisia tridentata*, sage grouse.

INTRODUCTION

Monoterpenoids (volatile or essential oils) have bacteriostatic and bactericidal activity (Nicholas, 1973; Nagy and Regelin, 1977). Before the discovery of antibiotics, microbiologists had studied these compounds to determine their usefulness as prophylactic agents. Because of the antimicrobial nature of monoterpenoids, researchers in the sixties and seventies became concerned about the possible adverse effects terpenoid-producing plants such as big sagebrush (*Artemisia tridentata*) might have on ruminant and cecal microorganisms' cellulolytic digestive abilities (Nagy et al., 1964; Oh et al., 1967, 1968; Dietz and

¹The use of trade or firm names in this paper is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

Nagy, 1976; Wallmo et al., 1977). These concerns ignored two important points. The first was the large amount of big sagebrush consumed by wintering wildlife species such as mule deer (*Odocoileus hemionus hemionus*), pronghorn antelope (*Antilocapra americana*), pygmy rabbit (*Brachylagus idahoensis*), and sage grouse (*Centrocercus urophasianus*) without any apparent harmful effects (Leach, 1956; Severson et al., 1968; Kufeld, 1973; Tueller, 1979; Green and Flinders, 1980; Remington and Braun, 1985). The second was the volatile nature of monoterpenoids.

Detrimental effects on wintering wildlife species as a result of heavy big sagebrush consumption has yet to be demonstrated outside the laboratory. Due to the high volatility of monoterpenoids, their levels in ingesta of wintering pygmy rabbits and mule deer were 23% and 20%, respectively, of expected levels (White et al., 1982b; Cluff et al., 1982). This would greatly reduce the harmful effects of the monoterpenoids on cellulolytic digestion by rumen or cecal microbes. One probable reason for the reduction is thorough mastication of the big sagebrush tissues by these animals. This would aid in the volatilization and loss of monoterpenoids from the digestive tract (Welch and Pederson, 1981; White et al., 1982b). But what about nonmasticating animals such as sage grouse, whose winter diets are nearly 100% big sagebrush leaves (Remington and Braun, 1985)? Have sage grouse evolved other types of mechanisms that reduce the levels of monoterpenoids in their ingesta, or have they evolved cecal microbes that are tolerant of monoterpenoids? The first step in finding answers to these questions is to demonstrate whether or not a reduction of monoterpenoids occurs in the ingesta. The purpose of this study is to test the hypothesis that the monoterpenoid levels in the ingesta from various digestive organs of sage grouse are less than expected from the big sagebrush leaves ingested.

METHODS AND MATERIALS

We used a two-phased procedure to test our hypothesis: (1) determine the monoterpenoid concentration of sage grouse ingesta from the various organs of the digestive tract, and (2) establish a baseline concentration of monoterpenoids in whole leaves of fed-on big sagebrush plants. For the first phase, we collected five wintering sage grouse from a flock feeding on a Wyoming big sagebrush (*A. t. ssp. wyomingensis*) flat about 15.6 km west of Loa, Utah (section 30, R2E, T27S). The birds were shot between 1200 and 1400 hours during mid-February 1984. We chose those times because the birds should by then be between their morning and evening feedings—when, we believe, the crop would be emptied and the gizzard full. After a bird was killed, it was frozen with dry

ice and placed in a cooler filled with snow for transporting to a laboratory freezer.

To obtain ingesta samples, the birds were thawed for about 8 hr. Breast muscles and sternum next to the keel along with other tissues were cut to allow removal of the windpipe, heart, lungs, liver, and the digestive tract. The digestive tract was isolated from the other organs and quickly washed in cold tap water. The mesentery was cut to allow for the separation of the various digestive organs. Ingesta were removed from the gizzard, duodenum, small and large intestine, and cecum. With the exception of the gizzard, ingesta from the other organs were pooled into one sample for each organ. At the time of pooling, we were concerned there would not be enough dry matter from individual bird organs to allow for extraction and monoterpene analyses. This proved to be true; however, enough dry matter was in the ingesta of the gizzards to allow separate bird analyses.

Ingesta were placed in beakers (250 ml) and thoroughly mixed. About 20% of the ingesta from each sample was removed for dry matter determination. The weights of the remaining ingesta were determined and placed in 250-ml Erlenmeyer flasks. Then 150 ml of absolute ether was added to the flasks to extract the monoterpenoids. The flasks were rotated in a shaker at 215 rpm for 2 hr. Separatory funnels were used to separate water from the ether extractions. Next, the extracts were concentrated to 20–30 ml by reduced pressure. One milliliter of a carvone standard (31.25 g/250 ml) was added. Absolute ether was then added to bring the volume of each sample to 50 ml. We stored extracts in airtight polyethylene bottles at -35°C until chromatographic analyses.

Chromatographic analyses were achieved with a 5830A Hewlett-Packard flame ionization, reporting gas chromatograph. Monoterpenoids were separated by use of a 120-cm \times 3-mm stainless-steel column packed with 10% Carbowax 20 M on 80–100 chromosorb WHP. Temperature programming (Table 1) was used to separate individual monoterpenoids. We identified monoterpenoids through retention times of standards. Dry matter content of each sample was determined, and the concentration of individual monoterpenoids was expressed as a percent of dry matter.

For the second phase, establishment of baseline monoterpene concentrations in the food, we collected vegetative samples in the same area where we had collected the sage grouse. The birds had been occupying this area for four to five weeks prior to sample collecting and had fed on most of the big sagebrush plants. Whole leaves were needed for establishing the baseline values. Because of the small size of the big sagebrush plants and heavy use by the sage grouse, there were not enough whole leaves per plant to allow individual plant monoterpene analyses. Instead, from 250 fed-on Wyoming big sagebrush plants, a single vegetative current year's stem with leaves was removed. The

TABLE 1. GAS CHROMATOGRAPHIC PARAMETER SETTINGS USED TO SEPARATE AND QUANTIFY INDIVIDUAL MONOTERPENOIDS^a

Parameter	Setting
Initial oven temperature	70°C
Initial time	1.0 min
Initial temperature rate	5.0°C/min
Programmed temperature rate changes	
At 2.5 min	1.0°C/min
At 5.5 min	10.0°C/min
At 13.5 min	25.0°C/min
Final oven temperature	200°C
Hold at final oven temperature	5 min
Flow rate	30 ml/min of N ₂
Attenuation	8
Injection port temperature	250°C
Flame ionization detector temperature	250°C
Maximum oven temperature	225°C

^a Area rejection mode was used to reject the solvent (absolute ether) peak.

collecting of vegetative samples occurred seven days after collecting the birds. All 250 stems were pooled in a plastic bag, frozen with dry ice, and transported to laboratory freezers in a cooler filled with snow.

We separated frozen whole leaves from stems with a pair of small surgical scissors. Detached leaves were frozen by placing them inside a 1-liter, stainless-steel, wide-mouth vacuum bottle filled with 500 ml of liquid nitrogen. After removal from the stems, the leaves were poured out of the vacuum bottle into a strainer. After the liquid nitrogen evaporated, the leaves were placed in a plastic bag, sealed, and stored in a freezer until needed for grinding.

Grinding of whole leaves was accomplished by placing the leaves inside the mortar of a steel, motorized mortar and pestle. The mortar and pestle were precooled twice with liquid nitrogen. Liquid nitrogen was then poured over the leaves and the leaves ground to a fine powder. After grinding, the leaf material was placed in a polyethylene bottle fitted with an airtight cap and stored in a laboratory freezer until needed for monoterpene analysis.

A Soxhlet extraction apparatus with absolute ether was used to extract the monoterpenoids. Ten grams of leaf tissue were placed in a cellulose Soxhlet extraction thimble. A fiberglass plug was placed on top of the sample to prevent spillage during the extraction process. Monoterpenoids were exhaustively extracted from the leaf tissues over a 6-hr period. Next, the volume of the extract was reduced to about 20–30 ml by reduced pressure. An internal standard of carvone was added as described, then the extract brought to 50 ml

volume and stored in a freezer until needed for chromatographic analyses. Analyses for leaf monoterpenoids were the same as previously described.

Unfortunately, due to the pooling of ingesta and leaf samples, statistical analyses could not be performed on this data set. Nevertheless, meaningful general comparisons between baseline monoterpenoid levels of leaves and levels in ingesta can be made.

RESULTS

Results of the monoterpenoid analyses are given in Table 2. From a qualitative and, in general terms, from a quantitative point of view the monoterpenoid profile of the leaves matches the monoterpenoid profile of ingesta from the gizzards. This supports the assumption that the plants sampled were the same as those on which the birds were feeding.

Of the 81 digestive tract observations of individual and total monoterpenoid concentrations, 32 observations showed no detectable amounts of monoterpenoids, 39 observations were lower than the baseline concentration of leaves, 2 were the same, and 8 above the baseline (Table 2). Loss of monoterpenoids occurs early in the digestive process. Reduction of total monoterpenoids of ingesta from the gizzards was 36% of the total monoterpenoids found in the leaves. Ingesta from the duodena and ceca contained no detectable amounts of monoterpenoids. Small and large intestine ingesta contained only 1 and 8%, respectively, of the monoterpenoids of leaves.

DISCUSSION

The data presented in Table 2 show that dramatic reductions in the level of monoterpenoids occur. The reduction starts early in the digestive process in the gizzard where big sagebrush leaves are ground into small fragments.⁴ Ceca monoterpenoid levels were below detectable levels, perhaps even entirely absent from the ingesta. At any rate, the probable impacts of big sagebrush monoterpenoids on ceca microbes' cellulolytic digestive abilities are nil. These observations and conclusions are supported by four reports: Welch and Pederson (1981), Cluff et al. (1982), White (1982b), and Foley et al. (1987).

During *in vitro* digestion trials, Welch and Pederson (1981) observed loss of monoterpenoids. They added specific amounts of α -pinene (one of the most

⁴Remington and Braun (1985) described the gizzard of sage grouse as a nongrinding gizzard. The gizzard receives nearly whole (90% or more) leaves from the crop and grinds the leaves into fragments. What they probably meant to say was that the gizzard of sage grouse is a nongrit grinding gizzard and not capable of breaking down hard foods such as seeds.

TABLE 2. COMPARISONS OF MONOTERPENOID LEVELS IN WYOMING BIG SAGEBRUSH (*Artemisia tridentata* ssp. *wyomingensis*) EATEN BY SAGE GROUSE (*Centrocercus urophasianus*) AND LEVELS IN DIGESTIVE TRACT ORGANS^a

	Percentage of monoterpenoids										Total
	1.07 ^b	α -Pinene	Camphene	4.84 ^b	1,8-Cineol	β -Thujone	Camphor	α -Terpineol			
Food	0.05	0.14	0.21	0.26	0.94	0.13	1.44	0.06			3.23
Gizzard 1	0.02	0.13	0.21	0.49	0.36	0.16	0.97	0.05			2.39
Gizzard 2	0.02	0.11	0.15	0.39	0.54	0.18	0.74	0.05			2.18
Gizzard 3	0.01	0.01	0.18	0.39	0.46	0.12	0.77	0.05			1.99
Gizzard 4	0.01	0.05	0.17	0.33	0.44	0.16	0.79	0.05			2.00
Gizzard 5	0.02	0.00	0.18	0.29	0.43	0.13	0.74	0.05			1.84
Duodenum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			0.00
Small Intestine	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00			0.05
Large Intestine	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.00			0.27
Ceca	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			0.00

^aData expressed on a dry matter basis. No statistical test could be performed on this data set. Duodenum, small and large intestine, and ceca samples were pooled from five sage grouse. Food sample was pooled from single branches taken from 250 fed-on plants (whole leaves only).

^bUnknown monoterpene; number represents retention time in minutes.

volatile monoterpenoids) and camphor (one of the least volatile) to three digestive tubes containing only buffer. After the first incubation period, they measured the monoterpenoid levels in the three tubes. They found that all of the α -pinene added to the flasks was lost through the one-way valves into the atmosphere. Camphor loss was 17.3%. Before extracting the two monoterpenoids from the flasks, the two researchers noted a white condensate ring around the neck of the three flasks. This condensate ring was about 70 mm above the surface of the digestion solution (in this case buffer) and was identified as camphor. They concluded that, first, the force that drove the two monoterpenoids from the solution was the heat of the water bath—in other words, normal body temperature is sufficient to volatilize monoterpenoids; and second, monoterpenoids in the rumen of mule deer could be reduced through mastication, rumination, eructation, and possibly by absorption and excretion. Cluff et al. (1982) reported an 80% reduction between the monoterpenoid levels in the rumen ingesta of wintering mule deer and the levels expected from the ingested forage. White et al. (1982b) reported similar reduction for stomach ingesta from wintering pygmy rabbits; they also reported large monoterpenoid losses during mastication. Foley et al. (1987) reported similar results for the greater glider (*Petauroides volans*) and brushtail possum (*Trichosurus vulpecula*) but discounted losses due to mastication.

Sage grouse consume big sagebrush leaves whole, and thus the only probable pathway for reducing monoterpenoids from the digestive tract of a non-masticating, nonruminating, and probably noneructating animal is through absorption, probably at the gizzard and either elimination or assimilation (Cook et al., 1952; Alexander and Chowdhury, 1958). Igimi et al. (1974) found that radioactive limonene, a monoterpene, rapidly disappeared from the stomach of rats. Also, Narjisse (1981) was unable to detect monoterpenoids in the rumen ingesta of goats 3 hr after direct infusion. Rapid absorption of monoterpenoids across the mucosa of the stomach and gizzard of both ruminants and hindgut fermenters is highly possible.

Another way that sage grouse could reduce the amount of monoterpenoids in the ingesta is through diet selection, that is, selecting big sagebrush plants that have lower concentrations of monoterpenoids. Remington and Braun (1985) suggest that wintering sage grouse preferred Wyoming big sagebrush (*Artemisia tridentata* ssp. *wyomingensis*) over mountain big sagebrush (*A. t.* ssp. *vaseyana*).⁵ Wyoming big sagebrush contained lower concentrations of mono-

⁵We do not fully accept the interpretation of the data presented in the Remington and Braun (1985) report concerning the preferential use of Wyoming big sagebrush over mountain big sagebrush by wintering sage grouse. Data in their Table 1 suggest two to one that the use of Wyoming and mountain big sagebrush is a function of occurrence and not palatability differences between the two kinds of big sagebrush. We believe their random sample method is biased because portions of mountain big sagebrush grow at the bottom of draws where sage grouse seldom feed. Consequently, part of their perceived preferential use could be due to feeding habit differences and not to palatability differences.

terpenoids than mountain big sagebrush. They did not detect differences in monoterpenoid concentrations among browsed, unbrowsed, and random plants within the two types of big sagebrush. Welch et al. (1983) reported that monoterpenoid content of various accessions of sagebrush taxa was not significantly related to wintering mule deer preference. Also, White et al. (1982a) reported no significant correlation between monoterpenoid content and dietary preference of pygmy rabbits for big sagebrush. The case for sage grouse selecting for big sagebrush plants lower in monoterpenoids is shaky, but still viable enough to need more investigation.

Beck and Braun (1978) reported that sage grouse gain weight during the winter months. This winter weight gain is probably due to the interaction of four factors: (1) the relatively high digestibility of winter tissues of big sagebrush (Welch, 1983); (2) the reduction to near zero of monoterpenoid within the cecum (this study); (3) the relatively large volume of the sage grouse cecum in comparison to other birds (Leopold, 1953); and (4) a probably high turnover rate of foodstuff (a guess).

In summary, data presented support our hypothesis that the monoterpenoid levels in the ingesta from various digestive organs of sage grouse are less than expected from the big sagebrush leaves ingested.

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CALCULATED EFFECT OF PULSED PHEROMONE RELEASE ON RANGE OF ATTRACTION

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Abstract—In order to assess the possible function of pulsed release of wind-borne pheromones, calculations were performed to predict the concentration pattern downwind of the source. Two patterns of pulsed releases (sinusoidal and instantaneous) were compared to a constant release pattern. In all three cases, the average rate of release was the same. Assuming the receiving animal needs only a momentary exposure to concentrations above threshold to respond, it is clear that sinusoidal release has a greater distance of detection than constant release and that instantaneous release is even better. The relative magnitude of the increase in range of detection depends on the ratio of average release rate to threshold concentration. Pulsed releases have a greater advantage when the threshold is high and the range of attraction is inherently short. Under these conditions, sinusoidal release can double the range of attraction and instantaneous release can increase it 10-fold. In contrast, with a low threshold and consequent long range of attraction, the pulsed patterns are lost and the increase in range is insignificant. Several testable predictions are derived from the hypothesis that the primary function of pulsed release is to extend the range and time over which a given quantity of pheromone can act.

Key Words—Pheromone, plume, pulsed release, moth.

INTRODUCTION

There has long been speculation about whether animals transmit information via temporal patterning of chemical release (Bossert, 1968). It has been shown that certain simple organisms such as slime molds and nematodes can make use of a temporal pattern of a diffusing stimulus (Robertson and Drage, 1975; Dusenbery, 1988). It has also been demonstrated that the females of certain

moth species release sex attractants in a pulsed manner (Conner et al., 1980) and that males respond better to fluctuating than to constant concentrations (Baker et al., 1985). Although many possible functions for this behavior have been suggested, there are few reports of tests of specific functions. Among these few are a report that pulsing increases the rate of release, although this function may be limited to species that have an unusually high concentration of pheromone (Schal and Cardé, 1985), and a report that there was not improved orientation to the plume from a pulsed source (Cardé et al., 1984).

A straightforward hypothesis of the function of pulsed releases of pheromone is that they extend the range of attraction possible with a given average rate of release. This hypothesis is based on the assumption that a male moth needs only momentary exposure to pheromone concentrations above threshold to know there is a female upwind. Consequently, the greatest range of attraction is the greatest range at which the concentration is above threshold, even if only for a brief time (Aylor et al., 1976).

METHODS AND MATERIALS

The specific questions addressed here are: how much can the range of attraction be increased by pulsed release, and is the increase biologically significant? Precise answers cannot be obtained because of the effects of atmospheric turbulence. However, if uniform flow without turbulence is assumed, some relatively simple calculations can give an indication of what is possible. Specifically, the results should give a good indication of the relative advantage of pulsed release, since concentrations in a turbulent flow should all be proportional to the concentration at the source.

The calculations are based on the following parameters: The diffusion constant is 2×10^{-5} m²/sec, which is appropriate for molecular diffusion of a small molecule in air; the wind speed is 0.5 m/sec; and the pulse frequency is 1 Hz, which is close to what is commonly found in moths (Conner, 1985). Three patterns of pheromone release are compared—all with the same average rate of release:

1. Constant release at a fixed rate. Calculations were based on equation 3 of Roberts (1923).
2. Pulsed release according to a sinusoidal pattern that goes to zero momentarily, i.e., 100% modulation. Calculations were based on Bossert's equation 10 (1968) with an extra factor of 2 in the denominator so as not to assume reflection from the ground. (A square wave pattern would give rise to similar results.)
3. Release in instantaneous pulses much shorter than the time between pulses. Calculations are based on equation 2 of Roberts (1923) for a single

pulse. The distribution for a sequence of pulses was determined by adding the contributions of neighboring pulses. This procedure is mathematically rigorous (Crank, 1975, p. 11). The equation assumes an infinitely short pulse but will apply reasonably well to any pulse that is much shorter than the time between pulses after enough time has elapsed for the pulse to start spreading by diffusion (about 0.001 sec for a source size of 0.3 mm).

The pattern of concentration distributions at various distances along the axis of the plume for the three different types of sources is shown in Figure 1. Within a few meters of the source, instantaneous release produces much higher concentrations than the other two types of release. The sinusoidal release produces maxima that are, at most, twice the concentration of constant release. A

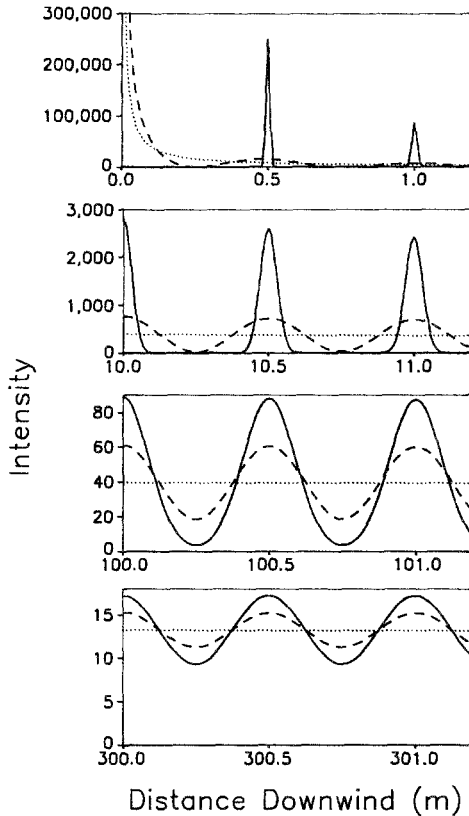


FIG. 1. Patterns of pheromone distribution. Intensity (sec/m^3) is concentration divided by the average rate of release. The solid line is for instantaneous release. The dashed line is for sinusoidal release. The dotted line is for constant release.

few hundred meters downwind, diffusion has smoothed out both types of pulsed releases so that the peaks are only fractionally higher than constant release. At this range, pulsing of the source makes relatively little difference.

The effect of pulsing on the detection distance is addressed directly in Table 1. The effect varies greatly depending on the ratio of threshold concentration to average rate of release. If the threshold is high so that the range of detection is inherently short, then pulsing can have a major effect. Sinusoidal release can double the range, and instantaneous release can increase the range an order of magnitude. If the threshold is very low, so that the range of detection is inherently long, then pulsing has very little effect on the range.

The actual ranges calculated should not be taken too seriously. In the natural environment, turbulence will tear the plume apart. The juxtaposition of pure air with air containing pheromone will increase the concentration gradients and speed up the diffusion process. However, it is now known that the use of "eddy diffusion constants" (based on average concentrations over several minutes) is a very poor approximation for biological receptors (that average over less than a second) because under natural conditions, concentrations downwind of a source fluctuate orders of magnitude in fractions of a second (Jones, 1977; Storebø et al., 1983; Murlis, 1986). However, the peak concentrations observed at any location should be proportional to the peak concentrations at the source. In view of this, the best interpretation of the above calculations is probably to consider them as setting an upper limit on what can occur in nature.

RESULTS AND DISCUSSION

The above calculations suggest that under certain circumstances pulsed releases can have a dramatic impact on the range of detection. If the primary function of pulsed releases is as hypothesized, a variety of predictions follow:

TABLE 1. MAXIMUM RANGE OF DETECTION

Threshold (sec/m^3) ^a	Range (m) for release patterns		
	Constant	Sinusoidal	Instantaneous
10,000	0.4	0.8	4.3
1,000	4.0	7.8	19.9
100	40	66	92
10	398	425	445

^aThreshold is measured in terms of the ratio of concentration to the average rate of release.

1. Pulsed releases should occur in species that have a relatively short range of communication based on amount released by the female and the sensitivity of male.

2. It should be possible for males to detect a pulsed source further away than a constant source with the same average rate of release. The data of Cardé et al. (1984) suggest pulsed sources with one third the average rate of release of a constant source were just as effective.

3. Males may respond under conditions where average concentrations are below threshold. This has been observed (Elkinton et al., 1984).

4. There is no reason to expect a pulsed plume to be easier to follow than a constant one. It has been proposed that a pulsed source might be easier to locate than a constant one, but tests have failed to demonstrate this (Cardé et al., 1984; W.E. Conner, personal communication).

5. The optimal pulse rate is probably of the order of magnitude of 1 Hz. The faster the rate, the faster diffusion evens out the pulses and the shorter the range over which pulsing is an advantage. Also, there is no point in pulsing faster than the adaptation time of the male's receptors, which is probably about 0.1 Hz. On the other hand, the slower the rate, the harder it will probably be for males to follow the plume. The optimum is probably close to the time scale of natural fluctuations due to turbulence in the atmosphere. This is in the range 0.1–1 sec (Murlis, 1986), which is also the range of pulse rates that are commonly observed (Conner et al., 1985).

6. There would be no purpose served by temperature compensation of the pulse rate. Therefore, one would predict that it would vary with temperature, as do most physiological processes ($Q_{10} \approx 2$). This has been observed (Conner et al., 1985).

7. It is not clear whether pulse rate should be expected to be adjusted for wind speed. Assuming the source is fixed in position, in different winds, pulse rate might be adjusted to maintain constant spacing between pulses (to maintain a fixed range at which the pulse pattern damps out or to maintain a maximal distance between peaks at which male orientation is successful). To do this, the pulse rate should be proportional to wind speed. This is approximately observed for *U. ornatrix* (Conner et al., 1985). Other species do not show a change in pulse rate with wind speed. However, those that call at dusk (when wind speed is relatively high) seem to pulse at a rate nearly twice that of those that call at dawn (when wind speed is generally lowest).

8. It is advantageous to release pheromone in short pulses in contrast to sinusoidal or square-wave patterns. To the extent this is physiologically practical, this pattern should be observed, although there is no advantage to pulses shorter than the adaptation time of the male receptors. Extreme cases of pulsed release might give males problems in locating the female at close range. How-

ever, at close range, constant release of a very low level (say 1%) would solve the problem. Thus, one might predict that the optimal pattern of release is short pulses superimposed on a low level of constant release.

9. There should be a limiting relationship between threshold, rate of release, rate of diffusion, rate of pulsing, and wind velocity, such that the effect of pulsing is still present at the range of maximum detection.

Clearly it is possible to test the hypothesis that the primary function of pulsed release is to extend the range and time over which a given quantity of pheromone can act. There are already some observations that favor it over alternatives, but more experimental work is required.

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MEASURING PLANT PROTEIN WITH THE BRADFORD ASSAY

1. Evaluation and Standard Method

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Abstract—The suitability of the Bradford protein assay for measuring plant protein was evaluated and a standard method developed. The assay involves extraction of dried, fresh, or frozen plant material in 0.1 NaOH for 30 min. Replicate 100- μ l aliquots of centrifuged supernatant are assayed with 5 ml Bio-Rad Bradford dye reagent (Coomassie brilliant blue G-250) diluted 1:4 and containing 3 mg/ml soluble polyvinylpyrrolidone. Absorbance at 595 nm is recorded after 15 min against a NaOH blank. Samples are calibrated against a ribulose 1,5-diphosphate carboxylase-oxygenase standard in NaOH. Procedures for plant preparation, extraction stability, the effects of phenol removal and quinone formation, and assay recovery are evaluated. Assay absorbance stability and techniques for increasing absorbance stability are reported. Changes in protein quality are briefly discussed.

Key Words—Soluble protein, Coomassie brilliant blue G-250, binding, leaves, extraction, spectrophotometry, protein assay.

INTRODUCTION

Foliar nitrogen concentration is an important variable in many ecological relationships (Mattson, 1980; Scriber and Slansky, 1981; Gnaiger and Bitterlich, 1984; Slansky and Scriber, 1985). Protein, particularly ribulose 1,5-

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diphosphate carboxylase–oxygenase (RUDP), is often the dominant form of foliar N (Ellis, 1977). Other forms include inorganic N, free amino acids and polypeptides, nucleic acids, and secondary metabolites such as alkaloids, betalains, cyanide, and glucosinolates.

A number of methods are available for estimating protein concentrations. Some of these methods, such as Kjeldahl (Maynard and Loosli, 1969) or elemental analysis, are not protein-specific. Other methods have greater specificity for protein but were developed for animal tissues and physiological fluids where protein concentrations are high and concentrations of interfering components are low. In plants, protein N is in low concentrations (1–4%), and phenolics are ubiquitous. These compounds interfere with many of the assays that are based on reduction (i.e., copper reduction, Lowry et al., 1951; bicinchoninic acid, Smith et al., 1985; silver binding, Krystal et al., 1985; ultraviolet, Warburg and Christian, 1941/1942; biuret, Riegler, 1914). Other methods that use protein precipitation (i.e., TCA, Harborne, 1973; Bensadoun and Weinstein, 1976; amido black, Martin et al., 1985) or hydrolysis (ninhydrin, Marks et al., 1985; OPA, Peterson, 1983) involve multiple steps that are time consuming and have a number of other limitations.

An ideal method for estimating plant protein in ecological studies should be specific and sensitive to protein, insensitive to interference, cheap, rapid, simple, and compatible with field sampling. The Bradford protein assay (Bradford, 1976) seems to fulfill many of these requirements and is widely used for animal proteins (Bio-Rad Laboratories, 1985). The method is gaining popularity for ecological studies with plants (Hare, 1983; Faeth, 1985; Denno et al., 1986; Trumble et al., 1987). The assay depends upon the binding of Coomassie brilliant blue G-250 dye to protein to produce a complex absorbing at 595 nm. The dye exists in three forms; cationic, neutral, and anionic. Only the anion binds to protein. Anion is not freely present at the dye reagent pH but is generated in the presence of protein. The dye has specific binding requirements for macromolecules with specific reactive functional groups—primarily arginine and basic and aromatic amino acid residues. The dye will not react with free amino acids, low-molecular-weight polypeptides (less than about 6–10 peptides), or many other nitrogen-containing or proteinlike molecules (Compton and Jones, 1985). Protein must be solubilized for the assay. Here we evaluate the use of the assay for plant protein and present a standard method. In particular, we address methods of preparation of plant material, extraction of protein from plant tissues, and assay stability.

METHODS AND MATERIALS

Standard Method

1. Fresh or frozen plant material should be finely chopped and ground with mortar and pestle or blended in a centrifuge tube using a tissue homogenizer.

A ratio of between 10 and 100 mg fresh weight (FW) per milliliter 0.1 N NaOH (pH 12.8) should be used, depending on the approximate protein and water content [i.e., 1–50 mg dry weight (DW) equivalents/ml]. Use 25% of the NaOH volume initially for grinding and the remaining volume to wash material into a centrifuge tube. Frozen material may be ground in liquid nitrogen, the liquid N₂ allowed to evaporate, and the 0.1 N NaOH then added. Dried material should be ground in a Wiley mill or similar device (<60 mesh), then combined in a centrifuge tube with 0.1 N NaOH at a ratio of between 1 and 50 mg DW/ml, again depending on approximate protein content. Final samples should contain sufficient protein to be within the linear range of the assay (approximately 0.1–1.8 mg RUDP equivalents/ml).

2. Samples are agitated for 3 sec on a vortex mixer and left to extract for 30 min at room temp.

3. Samples are remixed (3 sec), centrifuged for 5 min at high speed (>5000g) on a bench centrifuge, and the supernatant solution is decanted and remixed (3 sec).

4. Aliquots (100 μ l) of three replicates per sample (CV is usually circa 10% with three replicates), or 100 μ l 0.1 N NaOH (blank) are each mixed (3 sec) in a test tube with 5 ml of 1:4 diluted Bradford dye reagent (Bio-Rad Laboratories) that has been filtered, stored, and diluted according to the Bio-Rad Laboratories Manual (Bio-Rad Laboratories, 1985), but has been modified by addition of 3 mg/ml soluble polyvinylpyrrolidone (PVP) (Sigma pharmaceutical grade; MW ~ 40,000).

5. After 15 min, an appropriate volume of each replicate is transferred to cuvettes (glass, quartz, or disposable polystyrene) or tubes. The absorbance at 595 nm is recorded against the dye reagent/NaOH blank using a single- or dual-beam spectrophotometer.

6. Samples are calibrated against a standard of RUDP (Sigma) in 0.1 N NaOH with three replicates of at least four sample concentrations that are within the linear range of the assay and that also span the anticipated range of sample protein concentrations.

7. If agglutination of dye-protein complex occurs before 15 min, rerun the assay using a one half or one fourth dilution of the original extract.

8. If agglutination before 15 min still occurs and/or if grinding and extraction of fresh samples proves difficult because of resins or oils, then consider acetone washing. Wash plant material with three aliquots of 100% acetone (1 ml/60 mg FW or DW equivalents). Centrifuge and discard supernatant, then extract with 0.1 N NaOH as above. Protein recovery should be determined by adding RUDP to some samples.

Methodology Used in Evaluation

With the following exceptions, methods were described in the standard methodology or in the results.

Plant Species. Field-collected or greenhouse-grown leaves or algal material of the following species were used to represent a diversity of plant taxa and foliar chemistry: *Cladophora* sp. (Bryopsidophyceae); *Matteuccia pennsylvanica* (L.) Todaro (Polypodiaceae); *Phragmites communis* Trin. (Poaceae); *Phaseolis vulgaris* L. var. NY State Light Red Kidney (Fabaceae); *Comptonia peregrina* (L.) Coult. (Myricaceae); *Tsuga canadensis* (L.) Carr. (Pinaceae); *Citrus sinensis* (L.) Osbeck var. Valencia (Rutaceae); *Platanus occidentalis* L. (Platanaceae); *Quercus alba* L. (Fagaceae).

Preparation of Plant Material. Fresh material was weighed and processed immediately after collection. The FW to DW conversion was determined from independent samples for samples processed fresh or frozen. Plant samples for flash-freezing were collected, weighed, and immediately placed into liquid nitrogen or Dry Ice-acetone. Samples were stored in these solutions or used immediately. Some flash-frozen samples were lyophilized on a LabConco FD-3 or FD-5 at $<25\ \mu\text{m}$, $< -50^\circ\text{C}$, and then reweighed. Air-dried samples were first weighed fresh and then dried to constant weight at 24°C .

Comparison of Plant Preparation Methods. Single leaves of *P. occidentalis*, *C. peregrina*, *C. sinensis*, or *M. pennsylvanica* were split in half longitudinally along the mid-rib and the paired leaf halves compared. *P. communis* leaves were split in half horizontally across the mid-rib and the two halves compared. In addition, for all species, leaves were collected and mixed either by selecting for uniform size, age, and position on the plant or by randomizing by these variables. Random subsamples were taken from the mixture after preparation.

Extraction of Plant Material. Between 20 and 300 mg DW or 400 and 800 mg FW was extracted with 10 ml of either phosphate buffer (0.1 M KH_2PO_4 , pH 7.0, or 0.2 M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ adjusted to pH 6, 7, 8, 9, or 10) or 0.1 N NaOH, pH 12.8.

RESULTS

Preparation of Plant Material. We compared protein yields and reproducibility of analyses between fresh material and flash-frozen, freeze-dried, and air-dried tissues, for nine species of plants. Species varied considerably in the yields obtained by the same preparation method, and different preparation methods gave very different yields with the same species (Table 1). Contrary to our expectations, fresh material did not always give the greatest protein yield; air-drying did not uniformly produce lower yields; flash-freezing did not consistently result in similar yields to fresh material. We noted that species that were difficult to grind—in particular, resinous *Tsuga canadensis* and tough *Platanus occidentalis*—gave greater yields when the preparation involved freezing or air-

TABLE 1. EFFECT OF PREPARATION METHOD FOR PLANT MATERIAL ON ESTIMATION OF PROTEIN CONTENT

Species	Range of protein content of fresh material, %FW (range of CV %) RUDP equivalents	Mean protein content as % of that in fresh material (CV%)		
		Flash-frozen	Freeze-dried	Air-dried
<i>Tsuga canadensis</i> ^a	0.5-1.0 (3.3-13.8)	183a (3.2) ^b	—	301a (5.9)
<i>Platanus occidentalis</i> ^a	0.06-1.4 (6.0-15.7)	480a (4.8)	—	582a (9.0) ^c
<i>Comptonia peregrina</i> ^a	1.9-4.7 (2.4-10.5)	89b (4.3)	69c (36.0)	83b (5.3)
<i>Cirsium sinensis</i> ^a	1.8-2.6 (19.8-20.5)	94 (21.1)	109 (18.7)	59a (34.1)
<i>Phragmites communis</i> ^a	1.8-2.4 (22.9-40.4)	123b (19.7)	14a (96.2) ^c	55a (44.4)
<i>Quercus alba</i>	0.7 (4.3)	—	—	46a (12.3)
<i>Matteuccia pennsylvanica</i>	0.5 (4.6)	—	—	68b (10.9)
<i>Phaseolus vulgaris</i>	1.8 (4.8)	—	—	97 (4.9)
<i>Cladophora</i> sp.	0.3 (23.0)	—	—	86 (5.1)

^aSamples at various times of the season (NY and CA) were used for 5 species. The remaining species were sampled in August.

^bMeans significantly different from fresh material, paired *t* tests; a, $P < 0.001$; b, $P < 0.005$, > 0.001 ; c, $P < 0.05$, > 0.01 .

^cCoefficient of variation significantly different from CV or fresh material; *F*-test; $P < 0.05$, > 0.01 . All other means and CV values are not significantly different from fresh; *t* tests or *F* tests; $P > 0.05$. Sample sizes were a minimum of three in each case, excepting *P. communis* ($N = 4$ or 24), and *C. peregrina*, freeze-dried or air-dried ($N = 6$).

drying. Both these processes make the leaves more friable and facilitate grinding and subsequent extraction.

Variation in protein yield between replicate samples was influenced by both processing method and plant species. The greatest range in the coefficient of variation (CV) occurred with fresh material across plant species (2.4–40%), and with *Phragmites communis* between preparation methods (19.7–96.2%) (Table 1). However, variation between replicates for a given processing method was significantly greater than that found for fresh material in only two of the 17 cases. We concluded that these preparation methods did not consistently change the amount of variation between replicate samples, compared to that of fresh material.

Extraction of Protein. We compared dye responses to extracts of three species of plants extracted with phosphate buffer (pH 7.0) or 0.1 N NaOH (pH 12.8). Alkaline extraction gave significantly greater yields of protein than neutral buffer extracts, up to 35 times greater (Table 2). We also evaluated a range of pH values from 6 to 10 using phosphate buffer and pH 12.8 (0.1 N NaOH) with *C. sinensis*. Leaves were split in half vertically—one half being extracted at a low pH, the other half being the pH 12.8 control. Protein extracted, as a percentage of that removed at pH 12.8, was significantly less at all lower pH values (paired *t* tests; $P < 0.05$; $N = 4$), and increased at pH values above 8 (mean % ± 1 SD: pH 6 = 63 ± 4 ; pH 7 = 65 ± 4 ; pH 8 = 63 ± 8 ; pH 9 = 80 ± 3 ; pH 10 = 81 ± 1 ; pH 12.8 = 100 ± 2).

Extraction Time and Mixing. We evaluated the effect of the duration of alkaline extraction on protein yield with two species. There was no significant difference in protein yield for *Comptonia peregrina* extracted for 30 min to 24 hr (times = 0.5, 1, 2, 3, 4, 5, 24 hr; $N = 3$; paired *t* tests; controls = 0.5 hr;

TABLE 2. EFFECTS OF pH ON EXTRACTION OF PROTEIN

Species	Mean protein content (± 1 SD) ^a	
	pH 7	pH 12
<i>Tsuga canadensis</i>		
Fresh	0.36 (.11)	9.88 ^b (1.36)
Air-dried	1.43 (.28)	29.79 ^b (1.75)
<i>Platanus occidentalis</i>		
Fresh	0.53 (.35)	13.60 ^b (.82)
Air-dried	2.30 (1.52)	79.17 ^b (7.15)
<i>Comptonia peregrina</i>		
Fresh	1.41 (.03)	9.29 ^b (.20)

^aProtein content as mg/g FW or DW RUDP equivalents, depending on preparation method.

^bMeans significantly greater than mean for pH 7; *t* tests; $P < 0.0001$; $N = 3$ in all cases.

$P > 0.05$). Similarly, there was no significant difference in yield for *Citrus sinensis* extracted for 1–60 min (times = 1, 15, 30, 60 min; $N = 4$; paired t tests; controls = 1 min; $P > 0.05$). Since extraction is rapid when samples are finely ground, mixing of samples during extraction did not increase yield. Protein yields from alkaline extracts of *C. peregrina* mixed continuously for 30 min were not greater than yields for extracts that were initially mixed for 10 sec only (one-tailed t test, $P > 0.05$).

Stability of Extracts. We determined the stability of dye response to alkaline solutions that were extracted, centrifuged, and the supernatant refrigerated for periods between 0 and 30 days. We used bovine serum albumin (BSA) (Bio-Rad Laboratories) and RUDP standards and extracts of *Comptonia peregrina*. There was no significant difference in dye response in any case (RUDP: 0, 9, 30 days; $N = 3, 10, 5$, respectively; paired t tests; controls, RUDP at 0 days, fresh standard; $P > 0.05$. BSA and *C. peregrina*: 0, 14, 73 days; $N = 3$; paired t tests; controls 0 hr; $P > 0.05$).

Pretreatment of Plant Material with Acetone. This has been commonly used to remove phenolics and pigments (Loomis and Battaile, 1966; Eze and Dumbroff, 1982). We evaluated the effects of washing fresh plant material with 80% and 100% acetone prior to alkaline extraction, using five species of plants and pure RUDP. Acetone washing reduced protein yield in eight of nine experiments, including RUDP, with significant reductions in six cases. Yield was never increased (Table 3). We analyzed the acetone wash for the presence of protein, but could not detect significant amounts, even with pure RUDP. The decrease in yield was therefore probably due to a change in the solubility properties of proteins following acetone washing.

Addition of Phenol-Complexing Agents at Extraction. Soluble PVP or insoluble polyvinylpyrrolidone (PVPP) are often added to plant extracts to bind phenolics and prevent deactivation of enzymes at the lower pH of most extractions (Loomis et al., 1979). We evaluated the effect of adding these compounds to high pH NaOH-plant tissue mixtures at the time of extraction, using RUDP and *C. peregrina*. Addition of PVPP (Sigma, 10 mg/ml as a slurry) to RUDP standard in NaOH significantly reduced recovery (mean % recovery \pm 1 SD: RUDP = 98.6 ± 4.0 ; RUDP + PVPP = 77.2 ± 5.3 ; paired t test; $N = 3$; $P < 0.005$). Similarly, addition of PVP to alkaline extracts of *C. peregrina* reduced protein yield significantly in two separate experiments (mean protein content, mg/g DW RUDP equivalents \pm 1 SD: experiment 1 *C. peregrina* = 0.63 ± 0.03 ; *C. peregrina* + PVP = 0.54 ± 0.001 . Experiment 2 *C. peregrina* = 0.60 ± 0.01 ; *C. peregrina* + PVP = $0.52 \pm .005$; paired t tests; $N = 3$; $P < 0.005$).

Quinone Formation in Extracts. Extraction of plant material at pH 12.8 can result in the formation of brown-colored extracts. If phenols are present, these become oxidized to brown quinones. These quinones can then oxidize

TABLE 3. RECOVERY OF PROTEIN AFTER ACETONE WASHING.^a

Species or standard	Mean recovery of protein as % response of controls	
	80% acetone wash	100% acetone wash
<i>Comptonia peregrina</i>		
Fresh	45a ^b	98
Air-dried	—	101
<i>Tsuga canadensis</i>		
Fresh	88	—
<i>Platanus occidentalis</i>		
Fresh	57b	—
<i>Citrus sinensis</i>		
Fresh	65c	—
<i>Phragmites communis</i>		
Fresh	85	—
RUDP	86 ^c	80 ^c

^aControls were unwashed; $N = 3$ in all cases except *P. communis*; $N = 4$.

^bMeans significantly different from controls; paired t -tests: a, $P < 0.001$; b, $P < 0.005$, > 0.001 ; c, $P < 0.05$, > 0.01 . All other means are not significantly different from controls; t tests; $P > 0.05$.

^cRUDP means not significantly different from controls; χ^2 ; $P > 0.05$.

proteins (Loomis, 1969), forming melanoproteins (Pierpont, 1969a,b; Wehr and Loomis, 1971). We investigated whether or not quinone formation and presence affected the dye response to alkaline extracts. Addition of hydroquinone (HQ, 1.4 mg/ml) to phenol-free pure RUDP in alkaline extracts (pH 12.8) (1.4 and 0.7 mg/ml) produced the characteristic brown color but did not significantly affect dye response compared to alkaline RUDP controls in two separate experiments (mean blank corrected absorbance ± 1 SD: RUDP, 1.4 mg/ml = 0.87 ± 0.02 ; RUDP + HQ = 0.83 ± 0.02 ; RUDP, 0.7 mg/ml = 0.41 ± 0.03 ; RUDP + HQ = 0.39 ± 0.06 ; $N = 3$; paired t tests; $P > 0.05$). Similarly, addition of hydroquinone (1.4 mg/ml) to alkaline extracts of *C. peregrina* (10 mg DW/ml) (that were presumably naturally low in phenolics because they were not brown in color) then produced the characteristic brown color, but did not significantly affect dye response compared to controls (mean blank corrected absorbance ± 1 SD: *C. peregrina* = 0.82 ± 0.03 ; *C. peregrina* + HQ = 0.83 ± 0.03 ; paired t test; $P > 0.05$).

Recovery of Protein in Extracts. We determined the recovery of RUDP standards in the alkaline extraction procedure with pure RUDP standard and

RUDP standard added to plant extracts. We also carried out repeated extraction of the residue left after the first extraction had been centrifuged in order to determine what percent of the total extractable protein was removed in the first extraction. Pure RUDP was recovered 98.6 and 100% in two separate experiments (not significantly different from expected; χ^2 ; $N = 3$ in each experiment; $P > 0.05$). Addition of RUDP to air-dried *C. peregrina* resulted in an average recovery of 98.6% (three experiments; $N =$ three replicates per experiment; χ^2 not significantly different from expected; $P > 0.05$). Between 66 and 100% of the total protein extracted in three sequential extractions was removed in the first extraction, depending upon species and preparation method (Table 4). All but one value exceeded 75%, and two sequential extractions invariably removed over 95% of all the protein that could be extracted in three sequential extractions.

Assay Absorbance Stability. The dye anion-protein complex is not formed instantaneously, and so absorbance values initially fluctuate (Bio-Rad Laboratories, 1985). We examined stability of absorbance values for RUDP and BSA standards over time. Absorbance values stabilized after about 5 min (< 10% variation in three repeated measures within a 1-min period) and remained stable for up to about 30 min. Sometime thereafter, agglutination and precipitation of

TABLE 4. RECOVERY OF PROTEIN FROM STANDARDS AND PLANTS^a

Species or standard	Mean % of total protein extracted in first extraction
RUDP	98.6
<i>Comptonia peregrina</i>	
air-dried + RUDP	98.6
Fresh	75.6
Flash-frozen	79.9
Air-dried	78.9
<i>Quercus alba</i>	
Air-dried	85.7
<i>Matteuccia pennsylvanica</i>	
Fresh	84.9
Air-dried	100
<i>Phaseolus vulgaris</i>	
Air-dried	84.0
<i>Cladophora</i> sp.	
Fresh	82
Air-dried	66

^aPercent extracted in first extraction compared to three sequential extractions. $N = 3$ samples; standard deviations were always less than 7%.

the complex takes place. We noted that this agglutination occurred more rapidly (< 15 min) with some plant samples, presumably because some plant constituents promote agglutination at the assay pH. For example, when we added tannic acid (5% DW) to alkaline extracts of RUDP standard (1.4 mg/ml), dye reponse to protein was not affected (paired *t* test; $P > 0.05$), but the time before agglutination occurred was reduced to less than 15 min.

We evaluated the effects of sample dilution as a means of increasing the time before agglutination occurred. Table 5 shows that agglutination was slowed substantially by diluting samples to one half and one fourth strength before assay. We also examined the use of soluble PVP in the dye reagent. If rapid agglutination was due to phenolics, PVP should bind these compounds at the reagent pH of 0.77 (cf. Loomis and Battaile, 1966) and slow down the rate of agglutination. Table 5 shows that agglutination is slowed by the presence of 3 mg/ml soluble PVP (MW \approx 40,000) in the dye reagent. Soluble PVP in the reagent did not significantly change absorbance values compared to PVP-free controls ($N = 5$; paired *t* test; $P > 0.05$).

TABLE 5. EFFECT OF EXTRACT DILUTION AND SOLUBLE PVP IN DYE REAGENT ON AGGLUTINATION RATE OF DYE-PROTEIN COMPLEX^a

Sample dilution	Time (min)	Without soluble PVP		With soluble PVP	
		Mean blank-corrected absorbance	Agglutination ^b	Mean blank-corrected absorbance	Agglutination ^b
0	5	1.47	0	1.47	0
	10	1.34	+	1.47	0
	15		++		+
0.5	5	1.22	0	1.23	0
	20	1.20	0	1.22	0
	30	1.17	+	1.20	0
	45	0.97	++	1.13	+
0.25	5	0.87	0	0.87	0
	20	0.88	0	0.88	0
	30	0.88	0	0.87	0
	45	0.86	0	0.86	0

^aExtracts were of 40 mg/ml air-dried *Comptonia peregrina*. PVP was added at 3 mg/ml dye reagent. Missing absorbance values are due to the difficulty of measuring accurate values during agglutination.

^bAgglutination is scored none (0), slight (+), and extensive (++)

DISCUSSION

Our data show no simple, clear-cut differences in the effects of plant preparation methods. Flash-freezing usually gave higher yields than fresh material. This was most likely due to the ease of grinding frozen material relative to fresh plants. In fact, the effect of preparation method on the production of finely ground tissue may be more important than the effects of preparation method on leaf biochemistry. This may be because the NaOH extraction is very efficient at preventing or reversing complexing of protein and in solubilizing protein when material is finely ground. Air-drying seems to be an acceptable method in many cases, especially given the logistical advantages. Air-drying may be particularly appropriate when protein concentrations are low, since a large amount of plant material can be ground and extracted in the same NaOH volume, compared to fresh or frozen material. Freeze-drying is time-consuming, requires special equipment, and does not produce consistently better results than other simpler methods. Since there is so much variation between plant species in the response to preparation method, initial comparison of a chosen method against fresh material would be appropriate.

Extraction with 0.1 N NaOH appears to be an efficient, rapid process that avoids or reverses protein-phenol complexation. Extracts are stable, and quinone formation does not appear to affect dye-protein binding. We presume that extraction under alkaline conditions prevents the hydrogen bonding of polyphenolics to protein and the formation of insoluble complexes that occurs under neutral or acidic conditions (Loomis, 1969). Since the Bradford assay does not require that the solubilized protein be enzymically active, extraction at cytosolic pH—with all these attendant problems—can be avoided. Mattoo et al. (1987) have argued that the Bradford assay is unsuitable for plant tissues rich in phenols and phenolases, because free phenols, quinones, and polyquinones complex with protein and perhaps because these compounds also react with the dye-protein complex. However, their studies were carried out in unbuffered aqueous and saline solutions and not under the strong alkaline conditions we used. They also did not use PVP in the dye reagent. Using our techniques there is no evidence that phenolic-derived quinones cause any of these problems.

The alkaline extraction is also probably effective at solubilizing membrane-bound proteins, as well as cytosolic protein (Loomis, 1969), and is effective at leaching protein into solution away from cellular debris. This is evidenced by the rapidity of extraction and the lack of any effects of mixing extracts. This efficiency of extraction may partially compensate for any problems in grinding of plant material. For this reason, the standard method recommends a 30-min extraction, even though some plant species yield protein with less time.

It seems clear that acetone washing of plant material prior to extraction

reduces the yield of protein. Removal of phenolics by washing (Loomis and Battaile, 1966) may be unnecessary given the alkaline extraction procedure, and so there is little justification for the use of routine acetone washing. However, there are two potential benefits of acetone washing that should be considered for certain species. First, we observed that highly resinous species, such as *Tsuga canadensis*, became easier to grind after acetone washing had removed the resin. Acetone-washed samples were also easier to centrifuge because leaf particles were no longer caught in a resin-NaOH emulsion. Second, prior acetone washing reduced the rate of agglutination of the dye-protein complex for this same species. Thus if agglutination persists despite sample dilution and the use of PVP in the dye reagent, or if samples are resinous or oily, acetone washing—with appropriate corrections for changes in recovery—could be considered.

The addition of phenolic-binding agents, PVP or PVPP, to neutral or acidic extracts is common practice (Loomis et al., 1979). In our experiments, however, these compounds reduced protein recovery. At pH 12.8 PVP or PVPP will not hydrogen bond to phenols because the latter are ionized, but PVP or PVPP might cross-link protein and reduce its solubility, and this may explain the reduced yields we observed.

Rapid agglutination of dye-protein complexes only occurred with some species, and it would appear that sample dilution and the use of soluble PVP in the dye reagent can slow down agglutination sufficiently that the assay absorbance can be reliably determined after 15 min.

We believe the most appropriate protein standard is RUDP, since it is the most abundant plant protein, and large portions of the molecule are structurally very similar between species (Ellis, 1977). Therefore, variation in RUDP structure should not contribute greatly to variation in dye response between plant samples. Since the Bradford assay, like most other protein assays, measures relative protein concentration, differences in the composition of different proteins ("quality") between samples can result in differences in dye response (Bio-Rad Laboratories, 1985). Differences in protein "quality" between samples can be evaluated by using a TCA precipitation technique we have used. A ratio of 2:1 v/v of 5% (w/v) TCA to extract supernatant solution is used, and the samples centrifuged for 10 min at $\approx 12,000g$. The supernatant is discarded and the pellet dried and weighed. The pellet is then resuspended in the original volume of 0.1 N NaOH, and the assay carried out. Absorbance per unit dry weight of protein can then be compared between samples. Differences in this value can be used as an indicator of changes in protein "quality." We have observed 100% recovery of RUDP standard using this technique.

In summary, we consider that the use of alkaline extracts of finely ground plant material, followed by assay with PVP-modified Bradford reagent, is a simple, reliable, protein-specific assay for plant protein. The use of the alkaline

extraction procedure avoids many of the problems associated with extraction at lower pH.

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ACTIVITY OF QUASSINOIDS AS ANTIFEEDANTS AGAINST APHIDS

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Abstract—A series of quassinoids was tested for antifeedant activity against the aphid *Myzus persicae* (Hemiptera, Aphididae). Isobrucein B, brucein B and C, glaucarubinone, and quassin decreased feeding at concentrations down to 0.05% and isobrucein A was effective at 0.01%. Only quassin showed no phytotoxic effects and is therefore the most promising compound for further development.

Key Words—Antifeedant, *Myzus persicae*, Hemiptera, Aphididae, quassinoid, Simaroubaceae.

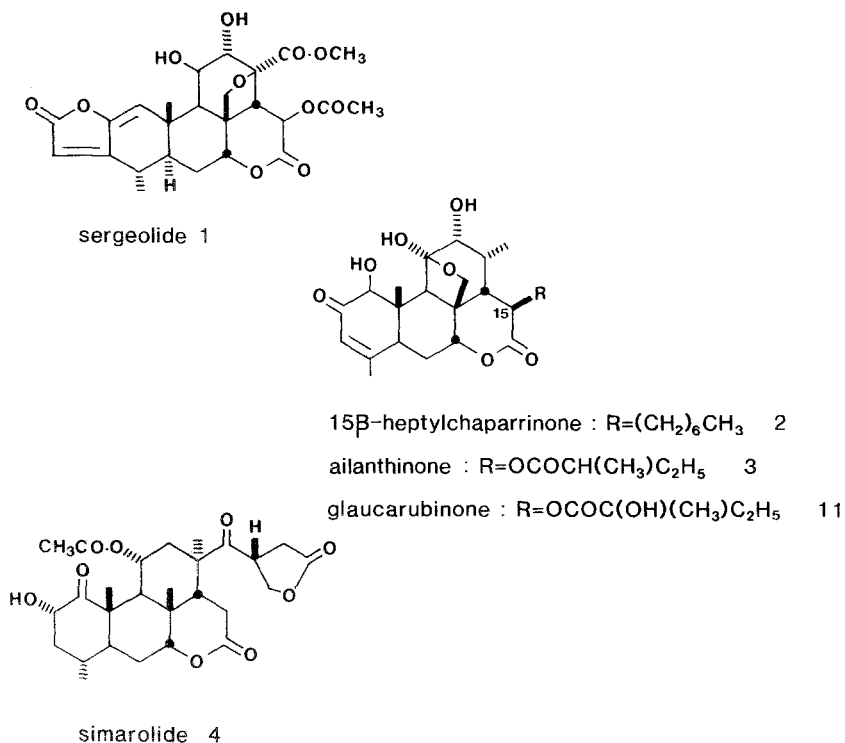
INTRODUCTION

Quassinoids (Bhatnagar et al., 1987; Polonsky, 1973, 1985) have been isolated from a number of plants belonging to the family Simaroubaceae. These compounds have many interesting biological properties, for example, antineoplastic activity (Cassady and Suffness, 1980), antimalarial properties (Trager and Polonsky, 1981), antimicrobial activity (Giesbrecht et al., 1978), and amebicidal action (Casinovi et al., 1981). Quassinoids were also shown to have insecticidal, antifeedant, and growth inhibitory effects against the tobacco budworm, *Heliothis virescens* (F.), and the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Klocke et al., 1985), and decreased feeding by the Mexican bean beetle, *Epilachna varivestis* Muls., and the Southern armyworm, *Spodoptera eridania*

(Cram.) (Leskinen et al., 1984). All these species are chewing insects. The aim of the present work was to investigate the activity of quassinoids as antifeedants against aphids, which are important plant pests having a piercing and sucking method of feeding.

METHODS AND MATERIALS

The quassinoids were extracted from plants by methods already described (see references in Polonsky, 1973, 1985). Quassinoid analog **2** (Scheme 1) was a semisynthetic product (Bhatnagar et al., 1987). The compounds, established as pure by nuclear magnetic resonance spectroscopy (Polonsky et al., 1975), thus obtained were dissolved in ethanol for testing against aphids. Aphids select their food plants by first making a series of short-duration probes into the surface of the plant. Only if the results of these initial investigations are satisfactory, do they go on to make a deeper penetration which, typically, terminates in the phloem. Antifeedant activity was assessed, therefore, by painting the



SCHEME 1.

upper (adaxial) leaf surfaces of Chinese cabbage, *Brassica campestris* var. *Chinensis* (L.) Makino, with test solutions and exposing the surfaces to aphids. The test chemical was applied to one side of each leaf lamina and the lamina on the other side of the midrib was treated with solvent only, to act as a control. Aphids were confined on the leaf surface under glass Petri-dish lids placed centrally so that the insects had a choice between equal areas of treated and control surfaces. Tests were done in dim light with leaves oriented randomly to eliminate possible effects of differential lighting. The aphids used in the tests were apterous viviparae of *Myzus persicae* (Sulz.) (Hemiptera, Aphididae), collected from cultures maintained on Chinese cabbage at 20°C and 16 hr day length. There were 20 aphids in each Petri dish and 10 replicated treatments per test. Numbers of aphids settled on treated or control areas were counted at 24 hr; wandering aphids and those on the midrib were not counted. Results were analyzed by analysis of variance. All compounds were first tested at a concentration of 0.1% a.i., painted on the leaf surface at ca. 0.01 ml/cm² (250 ppm w/w of leaf). Compounds active at this concentration were then tested at successively lower doses (0.05, 0.01, 0.005%) until antifeedant activity ceased. Leaves were kept for several days after each test and examined for signs of leaf damage, and the nature of any phytotoxic symptoms was noted.

RESULTS

The six compounds listed in Table 1 were inactive as aphid antifeedants at 0.1% a.i. All except simarolide (4) caused leaf damage, the symptoms varying in severity from small dry patches on the leaf to extensive erosion of the leaf surface.

TABLE 1. QUASSINOID INACTIVE AS APHID ANTIFEEDANTS AT 0.1% (ca. 250 ppm)

Material	Mean No. aphids settled		P	Phytotoxicity
	Treated	Control		
Sergeolide (1)	8.5	8.7	NS ^a	+
15β-Heptylchapparrinone (2)	10.1	8.7	NS	+
Ailanthinone (3)	8.2	10.0	NS	++
Simarolide (4)	8.1	9.3	NS	-
Simalikalactone D (5)	7.6	11.2	NS	+
Brucein A (6)	5.2	7.1	NS	+

^aNS = treated and control not significantly different at probability 0.05; ++, considerable leaf damage; +, slight leaf damage; -, no detectable leaf damage.

TABLE 2. QUASSINOIDS ACTIVE AS APHID ANTIFEEDANTS

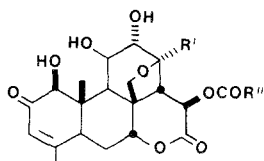
Material	Concentration (%)	Mean No. aphids settled		<i>P</i>	Phytotoxicity
		Treated	Control		
Isobrucein A (7)	0.1	3.3	11.0	0.001	++
	0.05	4.8	10.7	0.05	+
	0.01	5.4	11.0	0.05	+
	0.005	8.9	9.0	NS ^a	-
Isobrucein B (8)	0.1	3.7	12.0	0.001	+
	0.05	5.1	5.6	NS	-
Brucein B (9)	0.1	3.0	6.2	0.05	++
	0.05	7.4	8.5	NS	+
Brucein C (10)	0.1	2.8	7.4	0.01	++
	0.05	7.3	10.1	NS	+
Glaucarubinone (11)	0.1	4.1	12.4	0.001	+
	0.05	4.2	12.5	0.01	+
	0.01	7.5	9.6	NS	+
Quassin (12)	0.1	5.5	9.2	0.05	-
	0.05	4.8	9.2	0.01	-
	0.01	7.6	10.7	NS	-

^aFor explanation of symbols, see Table 1.

Of the more active compounds (Table 2 and Scheme 2), isobrucein A (7) exhibited antifeedant activity down to 0.01%, a dose that caused only slight leaf damage. Isobrucein B (8) and brucein B (9) and C (10) appeared less active, although with the latter two compounds at 0.1%, only a few aphids settled on either side of the midrib. Glaucarubinone (Scheme 1, 11) showed antifeedant activity accompanied by some leaf damage, whereas quassin (12) showed similar antifeedant activity with no visible effects on the plant.

DISCUSSION

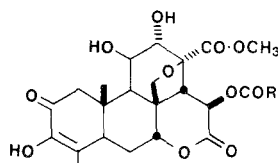
Although tests using leaf surfaces are useful in evaluating antifeedants for possible agricultural use, they do not distinguish between the direct action of antifeedants against insects and indirect effects involving changes in the leaf. However, there is evidence that the effects are separate; for example, sergeolide (1) and ailanthinone (3) caused considerable leaf damage but did not deter aphids



isobrucein A : $R' = \text{CO} \cdot \text{OCH}_3$; $R'' = \text{CH}_2\text{CH}(\text{CH}_3)_2$ 7

isobrucein B : $R' = \text{CO} \cdot \text{OCH}_3$; $R'' = \text{CH}_3$ 8

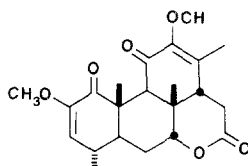
simalikalactone D : $R' = \text{CH}_3$; $R'' = \text{CH}(\text{CH}_3)\text{C}_2\text{H}_5$ 5



brucein A : $R = \text{CH}_2\text{CH}(\text{CH}_3)_2$ 6

brucein B : $R = \text{CH}_3$ 9

brucein C : $R = \text{CH} : \text{C}(\text{CH}_3)\text{C}(\text{OH})(\text{CH}_3)_2$ 10



quassin 12

SCHEME 2.

from settling, whereas quassin (**12**) was effective as an antifeedant but was not detectably phytotoxic.

There was no obvious correlation between chemical structure of compounds and antifeedant activity against aphids. Some compounds that were structurally similar, for example 15β -heptylchaparrinone (**2**) and ailanthinone (**3**), had a similar lack of activity, whereas other structurally very similar compounds, such as the isobruceins (**7,8**) and bruceins (**6,9,10**), had somewhat different antifeedant activities. Thus, in the isobrucein series, although a decrease in the size of the carbon chain of the acyloxy groups on carbon-15 involved a loss of activity (i.e., **7** to **8**), a similar change in substituents at carbon-15 in the brucein series (i.e., **6** to **9**) conferred activity. The increase in

hydrophilicity in the carbon-15 substituent between bruceins A (6) and C (10) also appeared to confer activity, and this was true on going from ailanthinone (3) to glaucarubinone (11). Isobrucein A (7), the compound most active against aphids, is also known to be active against chewing insects (Klocke et al., 1985). However, this correlation was not apparent for the other quassinoids tested here; for example, simalikalactone D (5) is relatively inactive against aphids but is known to be biologically active against the Mexican bean beetle and the Southern armyworm (Leskinen et al., 1984).

Phytotoxicity was displayed by many of the compounds tested and is a serious limitation to their use against phytophagous insects. In this respect quassin (12), being relatively active as an aphid antifeedant and not phytotoxic at the doses tested, is the most promising compound for further development.

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SAW-TOOTHED GRAIN BEETLE *Oryzaephilus surinamensis* (L.) (COLEOPTERA: SILVANIDAE)
Collection, Identification, and Bioassay of Attractive Volatiles
from Beetles and Oats

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Abstract—Over 200 beetle- and food-produced volatiles were collected from cultures of the saw-toothed grain beetle *Oryzaephilus surinamensis* (L.) on oats. It proved possible to develop the electroantennogram recording technique for these beetles, despite their small size, allowing volatiles causing antennal responses to be identified by coupled GC-EAG and subsequent GC-MS techniques. Three beetle-produced macrolide lactones were identified as (Z,Z)-3,6-dodecadien-11-olide, (Z,Z)-3,6-dodecadienolide, and (Z,Z)-5,8-tetradecadien-13-olide in an average ratio of 4.4:1:2. These have been reported as components of the aggregation pheromone from a different population of this species, although the ratio of the components produced was different. Three food volatiles with EAG activity were also identified: 1-octen-3-ol, 3-octanone, and nonanal. A mixture of the six identified volatiles produced similar levels of attraction, in a behavioral assay, to the entire mixture of collected volatiles.

Key Words—Aggregation pheromone, *Oryzaephilus surinamensis*, saw-toothed grain beetle, macrolide lactones, electroantennogram, GC-EAG.

INTRODUCTION

Oryzaephilus surinamensis (L.) is a pest of stored products on a worldwide scale and the most widespread pest of stored grain in the United Kingdom (Free-

man, 1976). Under present tolerance limits, consignments of grain can be rejected or downgraded following the discovery of a single live insect (Pinniger et al., 1984), so there is clearly a need for a system to detect and monitor levels of this species, especially at low densities. Traps containing sources of either food volatiles (Levinson and Levinson, 1979) or pheromones (Burkholder and Ma, 1985) have been proposed for stored-product insects in general, and a number of previous studies have attempted to identify suitable attractant materials for *O. surinamensis* (Freedman et al., 1982; Mikolajczak et al., 1983, 1984; A.M. Pierce et al., 1981; O'Donnell et al., 1983; Stubbs et al., 1985).

Observation of the behavior of saw-toothed grain beetles suggests the existence of a pheromone that promotes aggregation, since beetles often clump together in culture jars, either on the surface of the food or on the glass walls (P.R. White, personal observation). A chemical basis for this is suggested since, if beetle aggregations are removed from the glass walls, they often reform in exactly the same spot. Pierce et al. (1981) showed that both sexes of *O. surinamensis* aggregated in response to volatiles collected from adult beetles and also from frass, and, more recently, they have identified three compounds produced by male beetles, which together elicit responses in both sexes (A.M. Pierce, et al., 1984, 1985; H.D. Pierce, Jr., et al., 1984; Oehlschlager et al., 1987).

These previous investigations of both pheromones and food attractants have involved successive fractionation of the collected volatiles and subsequent behavioral testing. This method, however, suffers several drawbacks. To determine the response to every component in a complex mixture involves considerable effort. Furthermore, the presence of a repellent material in a fraction may mask the effect of an attractant. In contrast, this study aimed to collect both beetle- and food-produced volatiles and determine which were biologically active by coupled gas chromatography-electroantennography (GC-EAG) (Moorhouse et al., 1969) with subsequent behavioral assays of EAG-active components.

Although it has been widely used as a screening technique in Lepidoptera (Roelofs, 1984), the EAG has not previously been applied to silvanid or cucujid beetles. Electrophysiological recordings from *O. surinamensis* have been limited to preliminary studies of antennal contact chemoreceptors, although the olfactory receptors of the antenna have been the subject of a structural study (White and Luke, 1986). The aims of this study, therefore, were: (1) to determine whether it was possible to investigate the antennal response to volatiles by the electroantennogram method; (2) to collect all the volatiles produced by a culture of *O. surinamensis* on oats; (3) to investigate the antennal response to all the volatiles collected by the coupled GC-EAG technique and subsequently identify those materials causing EAG activity; and (4) to determine the behavioral response of *O. surinamensis* to the EAG-active volatiles, and thus

assess the relative potential of oat- and beetle-produced volatiles for use in detection traps.

METHODS AND MATERIALS

Insect Material. Insects for both the aerations and the assays were from a reference insecticide-susceptible strain, cultured on a 5:5:1 mixture, by weight, of rolled oats, whole-wheat flour, and brewers' yeast, at an average density of 4000 adults/kg of food, at 25°C and 70% relative humidity. Insects for behavioral assays were used when three to five weeks post-eclosion, and insects for electrophysiological recordings were four to five weeks after eclosion. In both cases, insects were removed from culture and deprived of food in clean glass tubes for 24 hr prior to testing.

Collection and Analysis of Volatiles. Volatiles were collected in a series of aerations conducted in a similar manner to that described by H.D. Pierce, Jr., et al. (1984). In each case, the all-glass apparatus was cleaned before use by heating at 510°C for at least 4 hr. Humidified, purified air was drawn at 2 liters/min through an aeration chamber, a trap containing Porapak Q, and a flowmeter. For the collection of beetle volatiles, each aeration started with about 10,000 *O. surinamensis* adults (estimated by weight) at about five weeks after adult eclosion, and 150 g of rolled oats. The aeration chamber was wrapped in foil to exclude light, and the temperature of the culture was continuously monitored by an internal thermocouple. To avoid collecting volatiles from larvae hatching in the aeration vessel, the food was renewed each week. Adults were removed using an aspirator and their number reestimated by weighing. After mixing with the appropriate proportion of fresh oats (0.015 g/adult), they were returned to the aeration chamber.

In the initial insect experiment the aeration chamber was 40 cm high × 6 cm diameter, and the majority of the insects congregated in one mass on top of the food. All subsequent aerations used shallower, wider chambers (20 cm high × 13 cm diameter) because in these there was a more even distribution of insects within the food and much larger amounts of volatiles were collected.

The Porapak Q (Millipore UK Ltd., Harrow, HA1 2YH, U.K.) was conditioned before use by rinsing with dichloromethane for 5 hr followed by Soxhlet extraction with HPLC-grade pentane for 48 hr. Each trap was 70 mm high × 20 mm diameter and contained about 14 g of the Porapak. (Tests had shown this was enough to prevent volatiles from an identical aeration bleeding out of the trap.) After collecting volatiles for periods of between two and five weeks, depending on the experiment, the Porapak trap was exchanged for a new one. The removed Porapak was extracted with 250 ml HPLC-grade pentane in a Soxhlet apparatus for 48 hr. (Tests had shown this to be sufficient to remove all these volatiles.) The extract was then concentrated to 1 ml on a rotary evap-

orator (16 mm Hg/20°C). Detailed results are presented here for the successive extracts (B1 to B5) from one typical aeration of beetles on oats, and for the single extract (F) from a control of 166 g of food (oats) alone.

Aeration extracts were analyzed on a Varian 3700 gas-liquid chromatograph fitted with a 25-m \times 0.22-mm-ID BP1 nonpolar bonded-phase capillary column (Scientific Glass Engineering UK Ltd., Milton Keynes, MK11 3LA). The carrier gas was helium at a linear flow rate of about 24 cm/sec. Injections were made in the splitless mode with the injector at 200°C. The oven program was 40°C for 1 min, then at 10°/min to 220°C, and the detector (FID) temperature was 240°C. The amplifier output was recorded on a model SP4270 computing integrator (Spectra Physics Ltd., St. Albans, AL1, 5UF).

Electroantennogram (EAG) Recordings. EAG responses were recorded as described in White and Birch (1987) with minor modifications. Intact individuals were used, mounted on a cork stage using double-sided adhesive tape. The recording electrode was inserted into the terminal (11th) antennal segment. Due to the toughness of the cuticle, small holes had to be punched with a finely etched tungsten needle prior to electrode insertion. The indifferent electrode was inserted into a hole punched in the second antennal segment.

To determine whether adult beetles produced any EAG-active volatiles, the EAG responses to combined beetle and food volatiles (extract B2) and food volatiles alone (extract F) were compared. A standard stimulus of 5 μ l of each extract was presented to 20 individuals (10 males, 10 females) in random order [5 μ l of extract F was equivalent to 280 gram hours (gh) of oats; 5 μ l of extract B2 was equivalent to 260 gh of oats plus 16,000 beetle hours (bh) of aeration]. Prior to each test, a solvent blank (5 μ l of pentane) was presented and the response subtracted from the following EAG response. Results were analyzed using a two-way analysis of variance (Sokal and Rohlf, 1969) to identify differences between the responses of individuals to the two extracts and between the responses of males and females.

Coupled Gas Chromatography-Electroantennography (GC-EAG). For each of the extracts the components causing EAG responses were located using a coupled GC-EAG technique (White and Birch, 1987), with oven conditions and programs as described above. Each extract was presented to a minimum of six individuals, using both sexes, and any GC peaks producing consistent responses in different EAG traces were subsequently identified by coupled GC-MS.

Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analysis was conducted on a Hewlett Packard 5790 gas chromatograph fitted with a 25-m \times 0.22-mm-ID CP-Sil 5 CB nonpolar bonded-phase capillary column (Chrompack UK Ltd., London E14 9TN), coupled to a Jeol DX300 mass spectrometer operating in electron impact mode at 70 eV. The carrier gas was helium at 8 psi head pressure, sample introduction was by splitless injection at 220°C and the oven program was 40°C for 1 min, then at 10°/min to 220°C.

Authentic Chemicals. Synthetic samples of (*R,S*)-(*Z,Z*)-3,6-dodecadien-11-olide (II), (*Z,Z*)-3,6-dodecadienolide (III), and (*R,S*)-(*Z,Z*)-5,8-tetradecadien-13-olide (IV) (Roman numeral notation as used by A.M. Pierce et al., 1984) were kindly supplied by Dr. A.M. Pierce, Simon Fraser University, Burnaby, British Columbia, Canada. They were quantified by GC using methyl laurate as external standard (chosen because of its similar polarity, GC retention time, and effective carbon number). Their purity was assessed as: *R,S*-II, 82% volatile material; III, 89%; and *R,S*-IV, 86%. Solutions of each lactone were tested using the GC-EAG technique. Only the lactones caused an EAG response (Figure 1), confirming that any impurities present would not affect the EAG results.

(*R,S*)-1-Octen-3-ol, 3-octanone, and nonanal (Aldrich Chemical Co. Ltd., The Old Brickyard, New Rd., Gillingham, Dorset, SP8 4JL, U.K.) were found to be pure by GC. In addition, two samples of enantiomerically enriched 1-octen-3-ol, 93%*R*:7%*S* and 17%*R*:83%*S*, were kindly supplied by Dr. D.R. Hall, Overseas Development of Natural Resources Institute, 56-62 Gray's Inn Road, London, U.K.

Behavioral Assays. The level of attraction to aeration extracts and synthetic materials was measured using a two-choice pitfall test (White and Birch, 1987; White, 1987), with 20 insects of mixed sex per test. Tests were run during mornings, and insects were tested once only and then discarded.

Behavioral responses were investigated to aeration extract B2 (beetle plus food volatiles), to individual components (synthetic) identified from extracts by GC-EAG, and to a synthetic mixture of these components in the amounts found in extract B2. Each extract, component, or mixture was tested at five dosages (using 5 μ l per test) with six replicates of each, in a totally randomized design. In a separate series of experiments, the response to 5 μ l of another extract of

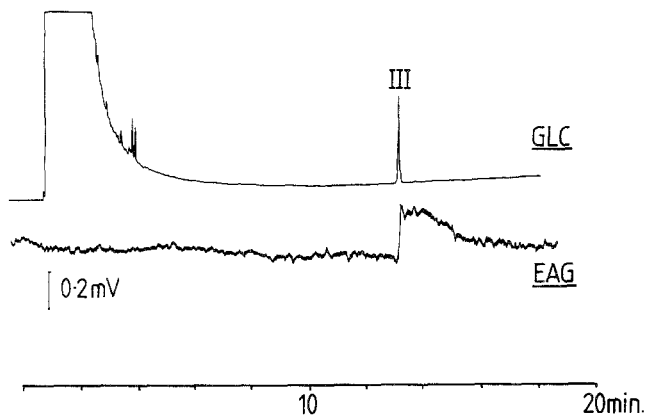


FIG. 1. Coupled GC-EAG trace for synthetic pheromone component III, showing lack of EAG response to impurities.

beetles on oats (A1) was compared to the response to a mixture of the three lactones alone, at the same dosages.

Results were expressed as the percent attraction to both test and control stimuli (number of insects found in each pitfall divided by the total number tested, $\times 100$), and differences between test and control were tested for significance using a χ^2 test.

RESULTS

GC Analysis of Aeration Extracts. GC analysis of the aeration extracts demonstrated over 200 volatile components produced by cultures of *O. surinamensis* adults on oats (Figure 2), and the parallel aerations of oats alone collected a similar number of volatiles (Figure 3). The aeration of oats produced between six and ten times the total GC area count (per microliter per day) of a parallel aeration containing neither oats nor beetles, while aerations from beetles on oats produced even more. The major source of the volatiles was therefore the oats or beetles, rather than the Porapak or the incoming air. Careful comparison of the reporting integrator outputs for the various extracts showed that, although the ratios of volatiles varied between extracts, only three volatiles, with relatively long retention times, were collected exclusively from cultures containing beetles (peaks C, D, and E in Figure 2) and were thus beetle-produced. The average temperature within the aeration medium was 24.6°C.

EAG Response to Aeration Extracts. Analysis of the results showed that the EAG response of individuals to a standard dose of extract B2 (beetle plus oat volatiles) was significantly greater than the response to F (oat volatiles alone) ($F = 55.5$, $P < 0.001$, Table 1). Adult beetles were therefore shown to produce EAG-active volatiles. There was no difference, however, between the responses of the sexes ($F = 0.1$ NS).

Identification of EAG-Active Volatiles. GC-EAG traces of the response to various aeration extracts of beetle plus oat volatiles each showed three major EAG responses and two minor responses (Figure 2). Similar response traces were obtained for both sexes. The three major responses corresponded to the volatiles that only appeared in beetle aerations (peaks C, D, and E), while the materials causing the minor responses (peaks A and B) were also collected from aerations of oats alone.

Analysis by GC-MS enabled the materials with EAG activity to be identified as follows:

Peak A: 1-Octen-3-ol:3-octanone (1:2) (quantified by examination of MS data), R_t (min) 6:04, m/e 110(4%) ($M-18$)⁺, 99(6), 85(10), 72(19), 57(100), and 41(17), identical to authentic 1-octen-3-ol and R_t 6:06, m/e 128(12%) M^+ , 99(46), 85(11), 72(53), 57(100), and 43(70), identical to authentic 3-octanone.

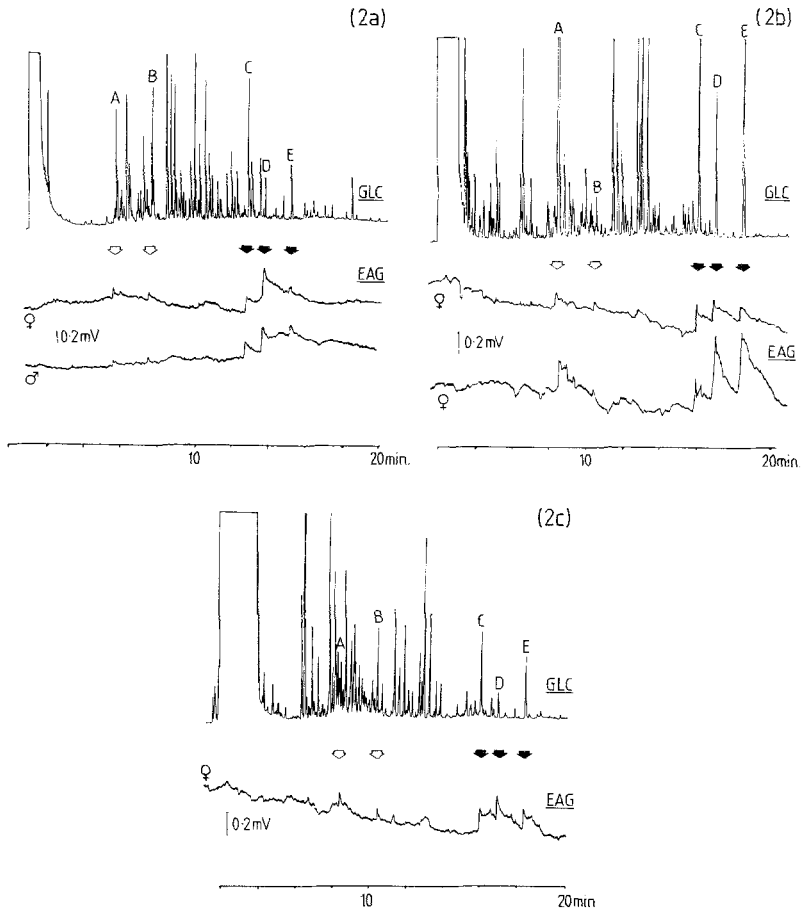


FIG. 2. Coupled GC-EAG traces for three aeration extracts from beetles on oats. Each trace shows three responses to beetle-produced volatiles (closed arrows) and two EAG responses to food-produced volatiles (open arrows.)

Peak B: Nonanal, R_t 11:09, m/e 124(8%) ($M-18$)⁺, 114(14), 98(60), 82(50), 70(60), and 57(100), identical to authentic nonanal.

Peak C: (*Z,Z*)-3,6-Dodecadien-11-olide (lactone II), R_t 13:08, m/e 194(13%) M^+ , 176(4), 134(14), 121(17), 93(35), and 79(100), identical to authentic lactone II.

Peak D: (*Z,Z*)-3,6-Dodecadienolide (lactone III), R_t 14:02, m/e 194(32%) M^+ , 166(11), 134(10), 121(11), 93(47), and 79(100), identical to authentic lactone III.

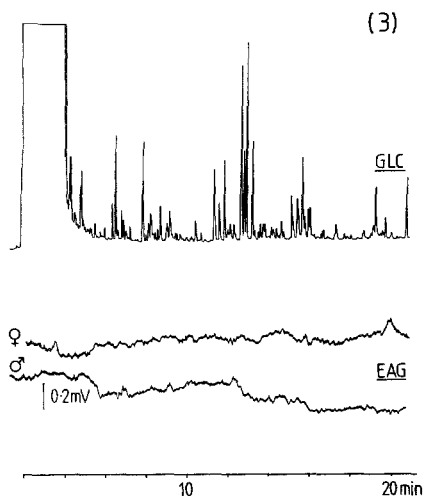


FIG. 3. Coupled GC-EAG traces for aeration extract F (oats only) showing no consistent EAG responses.

TABLE 1. EAG RESPONSES OF MALE AND FEMALE *O. surinamensis* TO 5 μ l OF AERATION EXTRACTS F (OAT VOLATILES) AND B2 (OAT PLUS BEETLE VOLATILES) ($N = 10$)

Extract	Amount	Response (μ V)	
		Males	Females
F	280 gh	88 \pm 28	75 \pm 20
B2	16,000 bh + 260 gh	392 \pm 78	368 \pm 46

Peak E: (*Z,Z*)-5,8-Tetradecadien-13-olide (lactone IV), R_t 15:27, *m/e* 222(19%) M^+ , 180(27), 140(24), 121(22), 93(52), and 79(100), identical to authentic lactone IV.

When the authentic chemicals were tested individually, both (*R,S*)-1-octen-3-ol and 3-octanone were found to have some EAG activity and were presumed to have contributed to the EAG activity of peak A in the aeration extracts. The EAG activity in each case was small, however, and when the two samples of enantiomerically enriched 1-octen-3-ol were tested, it was not possible to tell whether the EAG response was due to one or both of the enantiomers. Similarly, authentic nonanal was found to have a low EAG activity (Table 2).

GC-EAG traces of oat volatiles (F) failed to identify any EAG-active peaks,

probably because the EAG response to the whole extract was low (Table 1) and due to a summation of several small responses that could not be individually distinguished from background noise in the EAG recording (Figure 3).

Quantitation of EAG-Active Volatiles. Quantitation of the EAG-active volatiles in the aeration extracts was undertaken by GC comparison with methyl laurate in the case of the lactones, and with authentic material in the other cases. The concentrations of these components in extract B2, for example, were: lactone II, 142 ng/ μ l; lactone III, 44 ng/ μ l; lactone IV, 103 ng/ μ l; 1-octen-3-ol, 12 ng/ μ l; 3-octanone, 24 ng/ μ l; and nonanal, 71 ng/ μ l.

With regard to the beetle-produced volatiles, the chromatograms of various extracts (see, for example, Figure 2) showed that the amounts of lactones II, III, and IV varied between extracts, suggesting differing production rates between aeration cultures. Even within one culture the production rate seems to vary quite widely over time (Table 3). The ratio of lactones produced also varied both between and within individual cultures from 2:1:1 to 8:1:2. The mean ratio of lactones II, III, and IV in eight extracts was 4.4:1:2, respectively.

TABLE 2. EAG RESPONSE OF *O. surinamensis* TO 1 μ g OF AUTHENTIC CHEMICALS

Material	EAG response	N
(<i>R,S</i>)-1-Octen-3-ol	45 \pm 13 μ V	6
3-Octanone	80 \pm 26 μ V	6
Nonanal	90 \pm 26 μ V	6
(<i>R,S</i>)-Lactone II	168 \pm 21 μ V	20
Lactone III	232 \pm 25 μ V	20
(<i>R,S</i>)-Lactone IV	178 \pm 26 μ V	20

TABLE 3. PRODUCTION OF LACTONES IN CONSECUTIVE EXTRACTS FROM ONE AERATION

Extract	Average age after adult eclosion (weeks)	Average production rates (pg/bh)			Ratio II:III:IV
		II	III	IV	
B1	5-9	13.0	1.7	2.5	7.6:1:5
B2	9-12	44.4	13.8	32.1	3.2:1:2.3
B3	12-14	8.7	1.7	3.8	5.1:1:2.2
B4	14-17	40.6	12.1	28.6	3.4:1:2.4
B5	17-19	78.8	33.8	48.4	2.3:1:1.4

Behavioral Assays. The pitfall assay demonstrated that the aeration extract B2 was strongly attractive to adult beetles (Figure 4g), with a threshold equivalent to approximately 16 beetle-hours. When tested individually, the identified non-beetle-produced volatiles caused little evidence of attraction (Figure 4a-c). Neither 1-octen-3-ol nor 3-octanone were attractive at any of the doses tested, and at 100 ng 1-octen-3-ol acted as a repellent. Similarly, nonanal showed evidence of repellency at this dosage, yet was attractive at the highest dosage.

The three beetle-produced lactones all demonstrated attractancy, although to differing extents. Lactone III was only attractive at the highest dose (1000 ng), while the other two materials also produced significant responses at lower doses (Fig. 4d-f)

When the six identified components were combined to give the concentrations found in the aeration extract B2 (using twice the amounts of the chiral lactones II and IV, since only racemic material was available), the mixture produced high levels of attraction (Figure 4h), similar to that of the complete aeration extract. In the separate experiment, the attraction of beetles to 5 μ l of aeration extract A1 (beetles on oats) was $70.9\% \pm 3.5\%$ (control response subtracted from test response). The response to the mixture of the lactones alone at the same dosages (II, 13.9 ng; III, 4.0 ng; IV, 6.5 ng) was $61.4\% \pm 4.3\%$. These responses were not significantly different (χ^2 test).

DISCUSSION

Despite their small size, it proved possible to record EAG responses from the antennae of *O. surinamensis*. Furthermore, by use of the coupled GC-EAG technique, it was possible to screen all the aeration volatiles for EAG activity in a relatively short period. Examination of collected volatiles by coupled GC-EAG identified the same three active beetle-produced components previously reported by A.M. Pierce et al. (1984, 1985), who used a totally different method. This confirms the earlier findings and suggests that both our strain and the Pierce strain use the same compounds for intraspecific communication. H.D. Pierce, Jr., et al. (1984) and A.M. Pierce et al. (1987) also reported the production of small amounts of a fourth lactone, (*Z*)-5-tetradecen-13-olide, by males of their strain, but did not consider this to act as a pheromone component. Despite careful analysis of the aeration extracts, no evidence of this other material could be detected in the present study. Other differences in the pheromone components could also occur, however, since two of the components are chiral compounds (lactones II and IV), and separate strains of one species have previously been found to utilize different blends of optical isomers (e.g., *Ips pini*; Birch, 1984). The Pierce strain of *O. surinamensis* produces and responds to only the *R* enantiomers of lactones II and IV (Oehlschlager et al., 1987; A.M.

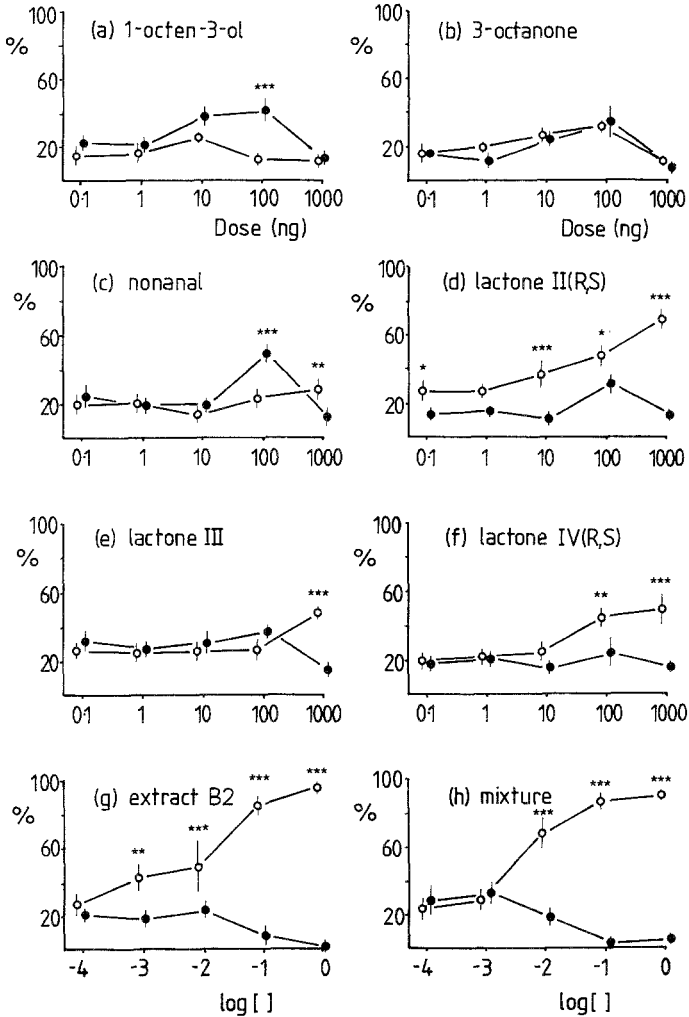


FIG. 4. The behavioral response of *O. surinamensis*, in the two-choice pitfall bioassay, to: individual authentic chemicals (a-f), aeration extract B2 (g), and to a synthetic mixture of the authentic chemicals in the concentrations found in extract B2 (h). Points on graphs give means \pm SE ($N = 6$) of percent attraction to test (open circles) and control (closed circles) tubes. Significance of difference between test and control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (χ -square test).

Pierce et al., 1987). So far it has not been possible to identify the chirality of the lactones produced in this study or to test the response to chiral compounds, but this will be attempted as suitable material becomes available.

A.M. Pierce et al. (1984) reported that the production rate of the lactones remained reasonably constant when the adult age exceeded five weeks post-eclosion, whether the aeration cultures had 20 or 100 beetles/g of oats. In the present study, however, with about 67 beetles/g oats, the production rates varied widely. Since both the availability of food and the density of beetles were kept approximately constant, and there is no evidence to suspect artifacts in either the collection or analytical procedures, these fluctuations may reflect synchronicity in pheromone production or release. Casual observations of the beetles while the aerations were in progress did suggest that some synchrony in behavior does occur. It would be wrong to read too much into this, since the present experiment was not intended to be a study of production rates, but it is interesting to speculate why beetles at these relatively high densities should produce any aggregation pheromone at all.

The two strains of *O. surinamensis* studied did differ, however, in the ratio of the macrolide lactones produced. The Pierce strain produced a ratio of 1:1:3 (II:III:IV) (A.M. Pierce et al., 1984), while that measured above had a mean ratio of 4:1:2, demonstrating a different major component in the two strains. Such strain or population differences have been reported previously in several species (e.g., the turnip moth *Agrotis segetum*; Lofsted et al, 1986). Since glyceride acids in the diet appear essential for production of the macrolide lactones (H.D. Pierce, Jr., et al., 1984), such differences in production ratios could reflect different availabilities of precursors for each lactone between the diets used in each study. In their proposed biosynthetic routes, however, H.D. Pierce, Jr., et al. (1984) suggest that all three lactones in *O. surinamensis* are produced from a common precursor, linoleic acid. If this is so, diet differences would not explain the observed differences in production ratios between the two strains. Furthermore, the present study also demonstrated variability in the produced ratio within a strain, which was fed the same diet throughout. Assuming that the collection efficiency was constant, this variability could have been due to either, or a combination of, two causes. Either individuals could alter the ratio that they produce, for example, with age, or different beetles may produce slightly different blends and the relative numbers of such individuals could alter over time. To distinguish between these possibilities requires collection of volatiles from individual beetles, rather than from large, high-density cultures, and such techniques are under development.

The other three EAG-active compounds identified from the aeration extracts appeared to originate from the oats. 1-Octen-3-ol and 3-octanone are known to be formed when grain is contaminated by mold (Kaminski et al., 1973). 1-Octen-3-ol is known to be an attractant for tsetse flies (Hall et al., 1984), and 3-octanone has been reported to be a component of the alarm pheromone of the

ant *Crematogaster peringueyi* (Fletcher, 1970), but there have been no previous reports of the effects of either of these chemicals on the behavior of stored-product insects. Nonanal has previously been identified in oat volatiles and tested against *O. surinamensis* in a pitfall assay, but no significant attraction was observed, even at the highest dose tested (100 μg) (Mikolajczak et al., 1984). Using an insect activity detector assay, O'Donnell et al. (1983) recorded attraction to *O. surinamensis* with nonanal at 0.02 μl (16.5 μg) and 0.1 μl (83 μg), but not at 1.0 μl (830 μg). In the present study, only the highest dose of nonanal (1 μg) appeared to attract the beetles. At the lower concentrations it was found to repel the insects, as did 1-octen-3-ol, while 3-octanone appeared to produce no observable effect on beetle behavior.

Since the three beetle-produced lactones alone could account for most of the attraction observed to complete aeration extracts of beetles on oats, they appear to have the greatest potential, of the EAG-active volatiles identified here, to augment trap catch in the field. Their use, however, may be limited by their stability, availability, and present high cost. Consequently, they will only be worthwhile as attractants if their effectiveness can be maximized, which in turn requires understanding of the mode of action of such materials in the natural chemical communication system of these insects. To improve this understanding was the objective of the following paper (White and Chambers, 1989).

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SAW-TOOTHED GRAIN BEETLE *Oryzaephilus surinamensis* (L.) (COLEOPTERA: SILVANIDAE)
Antennal and Behavioral Responses to Individual Components and Blends of Aggregation Pheromone

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Abstract—The antennal and behavioral responses of the saw-toothed grain beetle, *Oryzaephilus surinamensis*, to the three components of its male-produced aggregation pheromone were investigated. EAG recordings showed no differences between the responses of the two sexes to the synthetic pheromone components. In contrast, laboratory behavioral assays demonstrated marked differences between the sexes. More females than males were consistently attracted to mixtures of the synthetic components, and this bias appeared to be caused by one component in the blend. Altering the blend ratio resulted in changes in the ratio of the sexes attracted. Thus, if, as suggested by preliminary work, males vary the blend produced, this should alter the relative response of the sexes to the aggregation pheromone.

Key Words—Saw-toothed grain beetle, *Oryzaephilus surinamensis*, Coleoptera, Silvanidae, aggregation pheromone, electroantennogram, behavioral bioassay, blend ratio.

INTRODUCTION

The saw-toothed grain beetle, *Oryzaephilus surinamensis*, is an important and widespread pest of stored grain on a global scale, yet in large bulks of grain it is difficult to detect, especially at low density. Traps baited with pheromones have been proposed as a means to improve the detection and monitoring of stored-product insects in general (Burkholder and Ma, 1985), but to achieve this, the mode of action of such pheromones must be elucidated to ensure that these often expensive chemicals are used to best advantage and that the chances of detecting insects at low population densities are optimized.

Chemical communication in *O. surinamensis* utilizes beetle-produced volatiles that constitute a multicomponent aggregation pheromone (A.M. Pierce et al., 1984). This classification, although accurate, belies the complex mode of action of such chemicals.

Aggregation pheromones have been defined as "substances produced by members of either or both sexes, that induce members of both sexes to aggregate" (Borden, 1985). Several functions have been ascribed to such aggregations. In the aposematically colored beetle *Lycus loripes*, pheromone-mediated aggregations provide mutual protection against predation (Eisner and Kafatos, 1962), while among bark beetles that attack and kill previously healthy trees, large aggregations are necessary to overcome the defenses of the host tree (Birch, 1984). It has also been suggested that aggregation pheromones have been evolved as indicators to conspecific individuals of a suitable food source or habitat (Shorey, 1973; Borden, 1977), although it is difficult to explain the advantage to the emitter of attracting potential competitors to a food source, unless group feeding or conditioning of the habitat occurs.

Since in many examples only one sex produces the aggregation pheromone, it is likely that such pheromones have at least a partial sexual function related to mate location or courtship. In such cases, although both sexes respond, the pheromone might be expected to attract primarily the nonproducing sex and thus act in part as a sex pheromone. In the bark beetle *Dendroctonus brevicomis*, for example, colonizing females release *exo-brevicommin*, which preferentially attracts males, while males release *frontalin*, which attracts mainly females (Vité and Pitman, 1969). Similarly, field tests with several species of *Ips* have demonstrated that more females than males are attracted to the male-produced aggregation pheromone (Wood, 1972; Birch and Light, 1977; Lanier and Burkholder, 1974; Lanier and Wood, 1975). Among stored-product insects, female rust-red flour beetles (*Tribolium castaneum*) aggregate more than males in response to the male-produced chemical (Levinson and Mori, 1983).

Such sexual differences in the behavioral response to aggregation pheromones have often been reflected at the sensory level by sexual differences in the EAG responses recorded. In the above case of *T. castaneum*, for example, the EAG response of the females to the aggregation pheromone was found to be greater than that of males (Levinson and Mori, 1983). In other cases, however, such as *Dendroctonus frontalis*, no differences in antennal sensitivity to pheromone were found, despite observed differences in the behavioral response (Payne, 1970, 1971, 1975). In yet other examples, such as the boll weevil, *Anthonomus grandis*, sexual differences in antennal sensitivity have been found to some components of multicomponent pheromones but not to others (Dickens, 1984).

A.M. Pierce and co-workers (1984) reported that the aggregation pheromone of *O. surinamensis* was male-produced, and that both sexes were attracted.

Until recently, however (White et al., 1989), no recordings have been made of the EAG responses to the pheromone components. The initial aim of this study, therefore, was to investigate both the antennal and behavioral responses of the saw-toothed grain beetle to synthetic pheromone components and to identify sexual differences at either the sensory or behavioral levels.

The pheromone produced by the saw-toothed grain beetle consists of three components (A.M. Pierce et al., 1984; White et al., 1989), and such multicomponent systems are typical of beetles (Tumlinson, 1985) and insects in general (Silverstein and Young, 1976). Such systems may operate in two ways: either each component causes a separate behavioral response (and thus would constitute separate pheromones), or all components are required as a specific blend before a response can be elicited (i.e., a multicomponent pheromone). Most work on pheromone blends has been conducted on sex pheromones of moths. Several studies have suggested that different components affect separate behaviors in the approach of males to the calling females (Baker et al., 1976; Bradshaw et al., 1983). Other work, however, has shown that all of the components are needed, even the minor ones, for maximum sensitivity to the pheromone during each separate behavior and that the blend acts as an integral unit (Linn et al., 1984, 1986; Baker and Cardé, 1979).

In many such blends, the ratio of components confers species specificity on the pheromone, since many closely related species use the same components (Roelofs and Brown, 1982). In *O. surinamensis*, however, the ratio of components produced appears to be variable, both within and between populations (White et al., 1989). Consequently, this study also attempted to investigate the role of individual components and mixtures in the attraction of conspecifics and to determine the effect of changes in the blend ratio on the level of response.

METHODS AND MATERIALS

Insect Material

Insects were obtained from a reference insecticide-susceptible strain (cultured in the laboratory since before 1958), and cultured on a 5:5:1 mixture of rolled oats, whole-wheat flour, and brewers' yeast at 25°C in a reversed 8 hr:16 hr light-dark lighting regime (lights on 1800 hr, lights off 1000 hr). Insects for both electrophysiological and behavioral assays were used four to five weeks after adult eclosion and were removed from culture and kept in clean glass tubes (5 × 2.5 cm) without food for 24 hr before use.

Olfactory Stimuli

Synthetic samples of the three macrolide lactones identified from *O. surinamensis* (A.M. Pierce et al., 1984; H.D. Pierce et al., 1984) were supplied

by Dr. A.M. Pierce, Simon Fraser University, Burnaby, British Columbia, Canada. They were quantified by capillary GLC, using methyl laurate as external standard, and the purity determined as (*R,S*)-(*Z,Z*)-3,6-dodecadien-11-olide (lactone II) 82%, (*Z,Z*)-3,6-dodecadienolide (lactone III) 89%, and (*R,S*)-(*Z,Z*)-5,8-tetradecadien-13-olide (lactone IV) 86% (White et al., 1989).

Logarithmic dilution series were made up for each component in hexane (Gold label, spectrophotometric grade, Aldrich Chem. Co.), for use in both EAG and behavioral assays, and stored at -70°C when not in use. A food odor, "carob distillate," was used in EAG tests as a standard stimulus, to control for possible variability between preparations and within the life of a single preparation. Carob distillate was collected as an aqueous vacuum distillate obtained by heating pods of the carob tree *Ceratonia siliqua* (Leguminosae) (Stubbs et al., 1985). Previous recordings had shown that there was no significant difference between male and female EAG responses to this food odor (White, 1987).

Electroantennogram (EAG) Responses

The responses of male and female antennae to the three synthetic pheromone components were investigated using the EAG technique (White et al., 1989). Each individual was presented with nine doses of each pheromone component, each in 10 μl of solvent, in ascending order of concentration, interspersed with blank (10 μl of solvent only) and standard (10 μl of carob distillate) cartridges. The order of presentation of the pheromone components at each concentration was randomized. A total of 20 individuals (10 of each sex), all four to five weeks after adult eclosion, were tested. The results were normalized as a percentage of the standard response to carob distillate, and then analyzed using a split-plot analysis of variance (ANOVA) to determine sexual differences and differences between the responses to the individual pheromone components (Sokal and Rohlf, 1969). An analysis was also performed on the raw (i.e., unnormalized) data for comparison, but both analyses gave similar results.

Behavioral Assays

The responses of insects to synthetic pheromone components and mixtures were measured using the two-choice pitfall bioassay method previously described (White et al., 1989). Each test used 20 insects and was run for 1 hr at 25°C in darkness. At the end of this time, the number of insects in the test and control pitfalls and those not responding were counted. For comparison between tests, an index of attraction was used, calculated as percent attraction = $100(T - C)/N$ where T is the number of insects in test pitfall, C the number of insects in control pitfall, and N the total number of insects tested (Tamaki et al., 1971; Nara et al., 1981). Percentage attraction could thus vary from +100%

(all insects in the test pitfall) through 0% (equal numbers in test and control tubes) to -100% (all insects in the control pitfall).

Sexual Differences in Attraction to Aggregation Pheromone. Sexual differences were determined by testing the attraction of males and females, separately, to a mixture of synthetic pheromone components in the approximate ratio produced by beetles of this strain (White et al., 1989).

Large numbers of insects were cultured, removed on emergence as adults, their sex determined (Halstead, 1963), and kept in single-sex cultures at equal densities for four weeks before use. The percent attraction to the synthetic pheromone components II, III, and IV in the ratio of 8:1:4 (using racemic mixtures of II and IV) was determined over a range of five dosages on a logarithmic scale, with 10 replicates of each sex at each dose. The experiment was conducted as a fully randomized design and the results tested by a two-way analysis of variance to determine any sexual differences in the responses (Sokal and Rohlf, 1969).

Although the above experiment would identify sexual differences in attraction to the aggregation pheromone, it did not allow for differences in culture conditions prior to testing. As males produce the pheromone, females would not have been exposed to pheromone prior to the tests, in contrast to the males, and this could be responsible for any observed differences in their responses. To examine this possibility, therefore, a second experiment was conducted in which insects were removed from culture at emergence, sexed, and both single and mixed-sex cultures set up. These were tested as before after four weeks, but at just one dose of the synthetic pheromone mixture (1.0 μg). After testing, the insects in the mixed-sex assays were sexed to determine the percent response of each sex in each test. Thus, for the mixed-sex insects, the culture and test conditions were identical for both sexes, and so could not affect the behavioral response. Results were analyzed using a two-way analysis of variance (Sokal and Rohlf, 1969) to identify any effects of sex and culture type on the attraction to the pheromone mixture.

Attraction to Individual and Multiple Pheromone Components. For both this and the subsequent experiment, insects were raised as mixed-sex cultures, used when four to five weeks old, and sexed on the day prior to use. Using the two-choice pitfall assay, each sex was tested for its response to individual pheromone components, binary mixtures, and a mixture of all three components, with five replicates of each test mixture for each sex. To maintain the relevant ratio, the amounts of each component used were 0.8 μg (*R,S*)-II, 0.1 μg III, and 0.4 μg (*R,S*)-IV. The experiment was conducted in a randomized design, and analyzed using a factorial analysis of variance (Sokal and Rohlf, 1969) to determine the effect of the presence or absence of each separate pheromone component and the effect of the sex of the responding insects on the level of attraction observed.

Effect of Blend Ratio on Attraction to Aggregation Pheromone. To test the effect of blend ratio, each sex was tested separately against a variety of blends. The blends used were 8:1:4 [which corresponds to the average produced ratio of 4:1:2 assuming that both II and IV are produced enantiomerically pure (Oehlschlager et al., 1987)] and the five other permutations of this combination, plus ratios of 1:1:1 and 2:1:6. The same total amount of material was used in each test (1.3 μg), using racemic mixtures of lactones II and IV. [Recently, it has been reported that *O. surinamensis* produces and responds to only the *R* enantiomer of lactones II and IV (Oehlschlager et al., 1987; A.M. Pierce et al., 1987). If the strain used in this study also produces and responds to only one enantiomer of II and one of IV, this would have led to slightly different total amounts of active components in each of the blends tested. However, such differences would have been small on a log scale and would not have affected the male/female response ratio to any individual blend.]

The experiment was run as a randomized design with five replicates of each blend tested against each sex. Results were analyzed using ANOVA to identify significant effects of sex or blend ratio.

RESULTS

Antennal (EAG) Responses

Analysis of the results for all three lactones showed no significant differences between the responses of the sexes, using either the normalized ($F = 2.6$, NS) or raw EAG data ($F = 1.8$, NS) (Figure 1). There were significant differences, however, between the responses to the separate pheromone components ($F = 3.4$, $P < 0.05$ for normalized data; $F = 10.4$, $P < 0.001$ for raw data). The mean response to lactone III was higher than that to lactones II or IV, although when the higher doses were analyzed separately, there were no significant differences between the maximum EAG responses to the three pheromone components ($F = 0.7$, NS). (Mean response \pm SE to maximum dose (100 μg): lactone II = $267 \pm 42 \mu\text{V}$; lactone III = $247 \pm 29 \mu\text{V}$; lactone IV = $290 \pm 32 \mu\text{V}$; mean response to 10 μl of carob distillate = $480 \pm 33 \mu\text{V}$, $N = 20$).

Behavioral Responses

Sexual Differences in Attraction to Aggregation Pheromone. Analysis of the pitfall assay results (Figure 2) showed a highly significant difference between the sexes in their response to the synthetic pheromone mixture ($F = 23.9$, $P < 0.001$), with females showing the greater response at all doses above the threshold level of ca. 1 ng. The subsequent experiment on the effect of sex and culture type on the response also showed that more females than males were

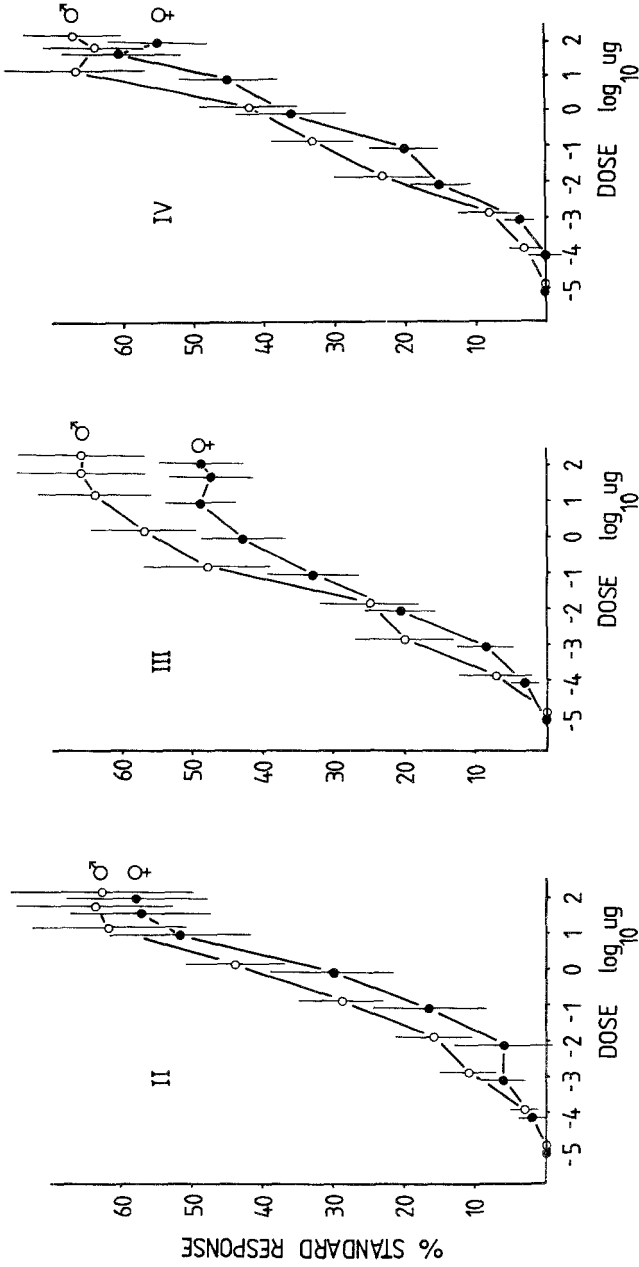


FIG. 1. EAG response of males (○) and females (●) to individual synthetic pheromone components R,S II, III and R,S IV (means \pm SE, n = 10).

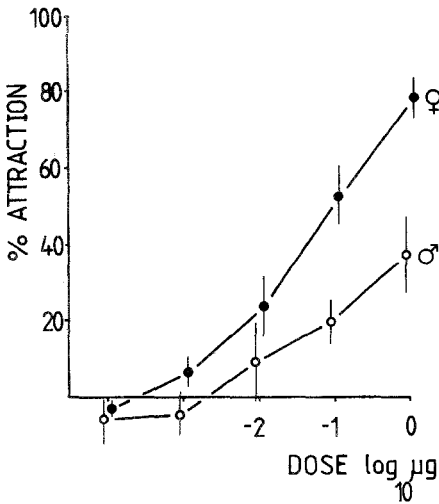


FIG. 2. Attraction of males (○) and females (●) to 5 doses of a mixture of the synthetic pheromone components R,S II, III and R,S IV, in the ratio 8:1:4, using the 2-choice pitfall bioassay. (20 insects/test, means \pm SE, $n = 10$).

attracted to the pheromone ($F = 46.5$, $P < 0.001$) (Table 1). Culture and test type were not significant as a main effect ($F = 1.4$, $P = 0.25$), but there was a significant interaction between sex and culture type ($F = 6.6$, $P = 0.01$). Inspection of Table 1 shows that fewer males were attracted in the single-sex tests than in the multiple-sex tests, while in females there was a slight bias in the opposite direction, accounting for the significant interaction.

Attraction to Individual and Multiple Pheromone Components. Statistical analysis of the results (Table 2) demonstrated that the presence of each of the three pheromone components had a highly significant effect on the level of attraction produced ($P < 0.001$). Inspection of the results (Figure 3A) showed that lactones II and III caused limited attraction either singly or together. When used alone, lactone IV produced no significant effect, but caused a marked

TABLE 1. EFFECT OF SEX AND CULTURE TYPE ON ATTRACTION TO 1 μ g DOSE OF MIXTURE OF SYNTHETIC PHEROMONE COMPONENTS (R,S)-II, III, AND (R,S)-IV IN BLEND RATIO OF 8:1:4.^a

	Male	Female
Single-sex culture and test	31.5 \pm 7.7	83.7 \pm 3.8
Mixed-sex culture and test (sexed after test)	52.3 \pm 5.8	76.0 \pm 3.1

^aFigures are percent attraction \pm SE. $N = 10$ for single-sex tests, $N = 20$ for mixed-sex tests, giving equal numbers of insects tested in each class.

TABLE 2. ANOVA TABLE SHOWING EFFECT OF INDIVIDUAL PHEROMONE COMPONENTS AND SEX OF RESPONDING INSECTS ON ATTRACTION TO INDIVIDUAL AND MIXTURES OF SYNTHETIC LACTONES.^a

Source	df	F
Sex	1	16.4***
Lactone II	1	29.4***
Lactone III	1	25.5***
Lactone IV	1	24.8***
Sex × II	1	29.2***
Sex × III	1	0.0
Sex × IV	1	3.1
II × III	1	3.8
II × IV	1	3.7
III × IV	1	9.2**
Sex × II × III	1	0.0
Sex × II × IV	1	5.6*
Sex × III × IV	1	0.0
II × III × IV	1	0.0

^aLevels of significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

increase in the attraction of both sexes when added to lactone III, [accounting for the significant interaction between lactones III and IV ($F = 9.2$, $P < 0.01$)] and an increase in the attraction of females, but not of males, when added to lactone II (accounting for the significant interaction observed between sex and lactones II and IV). The sex of the responding insects had a highly significant effect on the behavioral response to the components and mixtures ($F = 16.4$, $P < 0.001$), with more females than males responding overall. Inspection of the results (Figure 3A) and the highly significant interaction observed between sex and lactone II (Table 2) showed, however, that significant differences between the sexes were only found in response to mixtures containing component II. The results therefore suggest that although this material had a large effect on the attraction of females, it had much less effect on male beetles.

Effect of Blend Ratio on Attraction to Aggregation Pheromone. The analysis of variance showed that both blend ratio and sex of the test insects were highly significant as main effects on the level of attraction (blend: $F = 4.4$, $P < 0.001$; sex: $F = 25.0$, $P < 0.001$) (Figure 3B), again with more females than males responding overall to the pheromone.

The results have been replotted in Figure 4 to show the effect of the pheromone blend on the attraction of males and females. Two trends are shown. First, the level of attraction of both sexes appeared to be positively correlated with the amount of component IV in the blend (i.e., vertical axis of Figure 4).

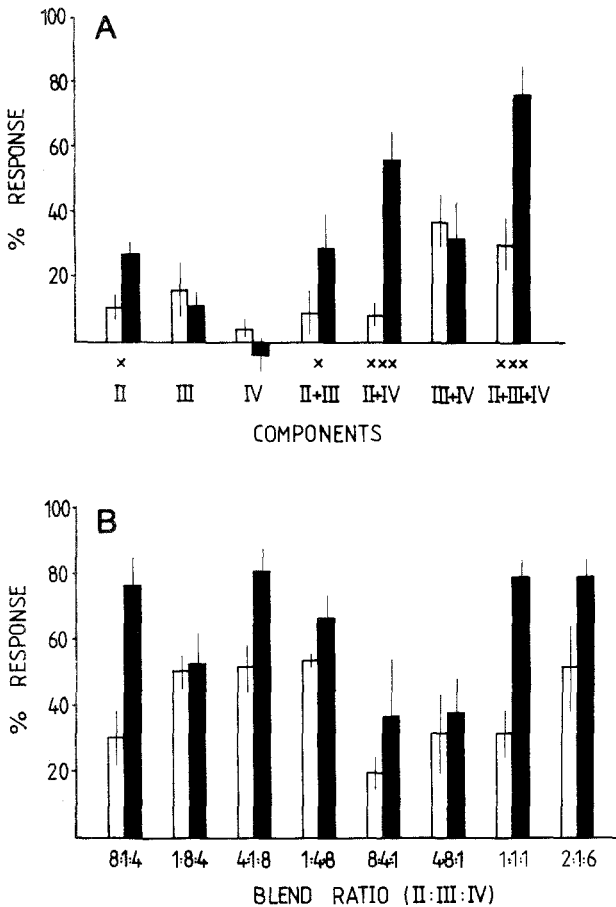


FIG. 3. (A) Attraction of males (open blocks) and females (closed blocks) to various combinations of the synthetic pheromone components (*R,S*)-II (0.8 μ g), III (0.1 μ g), and (*R,S*)-IV (0.4 μ g). (means \pm SE, $N = 5$). (B) Attraction of males (open blocks) and females (closed blocks) to various blend ratios of the synthetic pheromone components (*R,S*)-II, III, and (*R,S*)-IV, the total dose being 1.3 μ g in each case.

Secondly, the ratio of the male to female response (M/F) increased from left to right in Figure 4; i.e., as the amount of component II decreased and component III increased.

DISCUSSION

Although the sex producing an aggregation pheromone is often less sensitive, at the antennal level, to one or more of the components than its partner

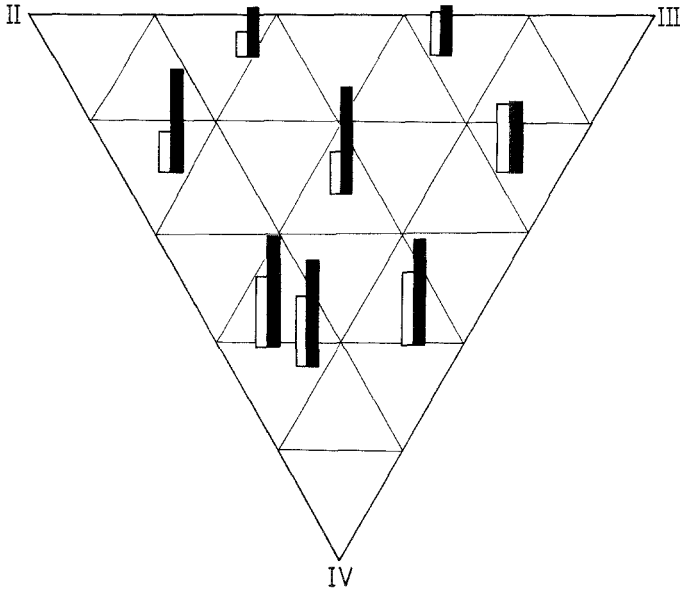


FIG. 4. Attraction of males (open blocks) and females (closed blocks) to various blend ratios of the synthetic pheromone components as shown in Figure 3B. The position of the base of the blocks corresponds to the blend ratio of the three components. Each corner represents 100% of the component indicated, falling to 0% along the opposite side of the triangle.

(Dickens, 1986), this does not appear to apply to *O. surinamensis*, since no sexual differences were found in either sensitivity or maximum EAG response to any of the synthetic samples of the three male-produced lactones.

The magnitude of the maximum EAG response has been suggested as a measure of the relative number of acceptor sites on the antenna for any given stimulus (Payne, 1975; Dickens and Payne, 1977). This assumes, however, that all acceptor sites contribute equally to the EAG potential recorded, regardless of their type or position. Sites for different components may differ in their transducing amplification. Also, even if transduction at each acceptor site produces an equal local change in membrane permeability, distal sites on the olfactory dendrites might be expected to make a smaller contribution to the EAG response recorded in the antennal hemolymph than more proximal sites. The above correlation, therefore, also assumes a uniform distribution of different acceptor site types on the dendritic branches of the olfactory cells. Given such assumptions, however, the results in this study imply that the numbers of acceptor sites on the antennae of male and female *O. surinamensis* for any one pheromone component were approximately equal. Similarly, since for any individual the maximum EAG amplitude produced by each component was roughly equal, acceptor

site populations for each component appeared to be of equal size. The apparent greater sensitivity of antennae to lactone III may be due to the optical impurity in the samples of lactones II and IV used, rather than differences in the acceptor populations.

The lack of sexual differences in the antennal response is in agreement with an earlier structural study, which found that there was no sexual dimorphism in either the numbers or distribution of antennal olfactory sensilla (White and Luke, 1986). Similarly, the small amplitude of the EAG responses to any of the pheromone components probably reflects the low numbers of olfactory sensilla (less than 200 per antenna) found in the same study.

Even the maximum EAG responses to the pheromone components were low, however, compared to the response to the carob distillate standard, suggesting that the antenna of *O. surinamensis* bears more acceptor sites for food volatiles than for the aggregation pheromone components. Such a situation is uncommon, since in most insect species studied the EAG responses to pheromone components have been found to be considerably larger. In the Douglas-fir beetle *Dendroctonus pseudotsugae*, for example, male and female antennae are 10–1000 times more sensitive to the aggregation pheromone components than to plant odors (Dickens, 1986). Some cases similar to *O. surinamensis* have been reported, however: male boll weevils (*Anthonomus grandis*), for example, are as sensitive to cotton volatiles as to any pheromone components (Dickens, 1984). The results in this study do, therefore, suggest the relative importance of food volatiles to adult saw-toothed grain beetles, and these may be involved in the location of suitable food sources, e.g., grain stores. In contrast to the antennal responses, however, there was a marked difference in the behavioral responses between the sexes to the synthetic pheromone components and mixtures. This would suggest sexual differences in the central integration of olfactory inputs and generation of motor output rather than differences in the sensory input from antennal receptors.

Although, as its name implies, the aggregation pheromone of *O. surinamensis* attracts both sexes, overall more females than males responded to the synthetic components and mixtures in all four experiments, suggesting that the male-produced blend does act in part as a sex pheromone. A.M. Pierce et al. (1984) also found a slightly greater response by females but attributed no significance to this difference. Millar and coworkers (1985a,b), however, found sexual differences in the responses of two related grain beetles, *Cryptolestes pusillus* and *C. turcicus*. In the latter species females showed the greater response, while in the former males responded at lower concentrations and had higher overall responses to the male-produced aggregation pheromone. This is inconsistent with the above hypothesis of a partial sex pheromone function in these grain beetles and merits further investigation. Another interesting result has been reported from *Oryzaephilus mercator*, the merchant grain beetle. Males

of this species produce a two-component aggregation pheromone (A.M. Pierce et al., 1984). While both components caused responses in both sexes, the results presented suggest that males were more sensitive to one component and that females were more sensitive to the other (A.M. Pierce et al., 1985). Thus, the ratio of the sexes responding to any mixture might be dependent on the blend ratio.

In *O. surinamensis* the female response to the naturally produced ratio was greater than that of males, whether the insects were raised in single- or mixed-sex cultures. Culture type did have an effect, however, since fewer males from single-sex cultures were attracted than from mixed-sex cultures, yet the reverse was true for females. The effect was apparently stronger for males. Culture type could affect insects in several ways, including mated state (virgins in single-sex cultures, mated insects in mixed-sex cultures) and differences in preexposure to the pheromone, which would only be present in male and mixed-sex cultures. The lower response of males from single-insect cultures than from mixed-sex cultures could be explained by greater preexposure to the pheromone in the former, but further work would be necessary to confirm this. The results do show, however, the importance of standardizing culturing methods and the mated state of insects used for assaying aggregation pheromones as well as sex pheromones.

Overall, although more females than males were attracted in all four experiments, the ratio of male to female responses varied with the components and mixtures tested. No mixtures, however, attracted significantly more males than females. The highly significant interaction found between the sex of the responding insects and the pheromone component II suggested that the greater response of females was largely due to the presence of this component. Addition of this material to a mixture increased the numbers of females responding, but had little effect on males. The tests with different blend ratios also supported this, since the ratio of males to females responding to a blend appeared to fall as the amount of component II in the blend increased. This contrasts with the antennal responses, however, since, at the peripheral sensory level, both sexes were found to be equally responsive to lactone II.

Since no detailed observations were made of the behavior of individual insects in this study, no conclusions can be drawn as to whether each component of the pheromone has a specific and separate effect on behavior or whether the response is to the entire blend. The results do show, however, that all three components are necessary to attract individuals most effectively, at least in females. If these materials are to be used in the field to improve detection of this species, all three materials would appear necessary to increase trap catch significantly. To optimize the effect of the pheromone, however, blend ratio must be considered, since alterations in the blend ratio had a clear effect on the level of attraction of both males and females.

Previous work on the importance of blend ratio has concentrated on the sex pheromones of moths. Such studies have suggested that the blend ratio of the sex pheromone confers species specificity on the signal (Baker et al., 1981; Linn and Roelofs, 1983) and that this is important in closely related sympatric species that utilize the same pheromone components. In *O. surinamensis*, however, blend ratio was not that critical, since several markedly different ratios produced equally high levels of attraction and, furthermore, the naturally produced ratio did not produce the greatest mean attraction in either sex. This contrasts with the above studies of moths, where departures from the natural ratio often resulted in sharp decreases in the male response. It should be noted, however, that the present tests were conducted with relatively high doses and that blend ratio may be more critical at concentrations around the threshold level for detection.

The blend ratio of the aggregation pheromone does not, therefore, appear critical for species specificity in these beetles. Specificity could be conferred by the chemical identity of the three components, since no other species is known to use this mixture, although *O. mercator* does use the same enantiomer of lactone II as a pheromone component (Oehlschlager et al., 1987). The actual blend ratio may be important in conveying extra information about the emitter, such as reproductive state, since both males and females appear to mate repeatedly, and this could explain the differential responses of males and females to changes in blend ratio. Such added complexity in aggregation pheromone communication systems might be expected since, not only are both sexes responding, but also insects that utilize aggregation pheromones are generally long-lived as adults (Burkholder, 1982) and may need to alter the "meaning" (Slater, 1983) of the signal over time. In the boll weevil *Anthonomus grandis*, for example, the aggregation pheromone attracts both sexes, but at one time of the year acts as a sex pheromone and attracts mainly females (Mitchell et al., 1972). In this case, however, the change appears to be in the responsiveness of the insects rather than in the blend ratio.

Collection of volatiles suggested that the blend ratio of the aggregation pheromone produced by *O. surinamensis* cultures varied both between and within populations, but it was not possible to distinguish whether this was due to individual males altering the blend they released or whether each male produced a fixed blend, and changes were caused by fluctuations in the numbers of males producing a given ratio (White et al., 1989). If males could alter the ratio they emit, theoretically they could affect the ratio of males to females likely to be attracted, perhaps drawing in females when sexually mature or both sexes if habitat conditioning (e.g., by raising humidity) was required. Further research awaits the development of techniques to collect and analyze the pheromone blend produced by individual beetles.

The present study has highlighted the complexity of the chemical com-

munication system in the saw-toothed grain beetle. If this system is to be successfully manipulated to improve detection and monitoring of this economically important yet elusive pest, a full understanding of the factors affecting both the production of and the response to the pheromone is essential.

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PYGIDIAL SECRETIONS OF *Pasimachus subsulcatus*
(COLEOPTERA: CARABIDAE) DETER PREDATION BY
Eumeces inexpectatus (SQUAMATA: SCINCIDAE)

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Abstract—The carabid beetle *Pasimachus subsulcatus* is an abundant ground-dwelling insect in west central Florida that exudes a powerful mucous membrane irritant when disturbed. This secretion can be sprayed over 10 cm from the abdominal tip. The southeastern five-lined skink, *Eumeces inexpectatus*, is an abundant insectivorous lizard sympatric with *Pasimachus*. We assessed the availability of *Pasimachus* to *Eumeces* and found it to be within the foraging microenvironment of the lizard. Analysis of *Eumeces* gut contents and field feeding trials indicate that *Pasimachus* are not ingested by the lizard, yet arthropods of comparable size and exoskeletal thickness are ingested. The movement response of *Eumeces* to isolated *Pasimachus* secretion constituents, conducted in a modified Y-maze laboratory experiment, was used to assess the repellent capabilities of the secretion. *Eumeces* are consistently repelled by *Pasimachus* secretion constituents, indicating that the beetle is protected chemically from the lizard.

Key Words—Defensive mechanism, avoidance response, *Eumeces inexpectatus*, *Pasimachus subsulcatus*, Coleoptera, Carabidae, lizard ecology, lizard predation.

INTRODUCTION

Noxious substances are among the myriad defensive mechanisms exhibited by insects. These substances are produced either internally (Smolanoff et al., 1975; Eisner and Dean, 1976; Eisner et al., 1976, 1978, 1981; Jefson et al., 1983; Carrel and Eisner, 1984) or obtained from the environment and incorporated into the defensive repertoire (Morrow et al., 1976). The mechanics of discharge

and the chemical constituency of certain arthropod defensive secretions have been studied extensively (Eisner et al., 1963a,b; Eisner and Meinwald, 1966). Arthropod secretions are effective deterrents to ants (Smolanoff et al., 1975; Eisner et al., 1976; Jones et al., 1976; Jefson et al., 1983), spiders (Eisner and Dean, 1976; Carrel and Eisner, 1984), and certain vertebrate predators (Eisner et al., 1978, 1981). Many species of carabid beetles possess defensive pygidial glands (Forsyth, 1972; Blum, 1982). Secretions of these glands contain formic acid, methacrylic acid, *p*-benzoquinone, toluquinone, *m*-cresol, and salicylaldehyde; all are noxious to vertebrate predators (Eisner, 1966). Information regarding the effectiveness of arthropod secretions against insectivorous reptiles, specifically lizards, is limited (Eisner et al., 1961; Vogel and Brockhusen-Holzer, 1984).

The carabid beetle *Pasimachus subsulcatus* is abundant throughout the year in the sandhills of west central Florida (McCoy, 1987). Both *P. subsulcatus* and the larger sympatric congener *P. strenuus* exude a highly volatile mucous membrane irritant from the pygidial gland when disturbed by a predator. The secretion is expelled either by a slow oozing over the surface of the abdomen or by an expulsion in a highly directional spray.

The southeastern five-lined skink, *Eumeces inexpectatus*, is an abundant, ground-dwelling lizard throughout the southeastern United States and is sympatric with *Pasimachus* in the sandhills of west central Florida. It occurs in both xeric and mesic habitats in central Florida. Skinks (family Scincidae) are grouped with the families Lacertidae, Teiidae, Cordylidae, Helodermatidae, Varanidae, in the Autarchoglossa (Camp, 1923; Bissinger and Simon, 1979). Autarchoglossans have a highly protrusible, elongated bifurcating tongue, a well-developed Jacobson's organ, and use chemoreception extensively for environmental assessment (e.g., prey selection and/or avoidance, search behavior, conspecific recognition, etc.), although vision may be equally important (Nicolletto, 1985; Cooper, 1981).

The congeneric five-lined skink, *Eumeces fasciatus*, ingests coleopterans; however, certain sympatric carabid beetles that were within the size range of ingested prey were absent from their scats and stomachs (Fitch, 1954). Forsyth (1972) surmised that the noxious qualities of certain carabid beetles protect them from predation.

How reptiles detect prey and avoid nonprey is poorly understood. Burghardt (1966) considered increased tongue-flick frequency by reptiles an indication of response to preferred prey; however, the specific motivation for tongue flicking may vary (see Simon, 1983). Experimental designs that focus on measuring predator movement in relation to potential prey may provide more conclusive evidence for understanding lizard predator-prey relationships than those that measure tongue flicking.

In this study, we quantify the movement responses of *Eumeces inexpectatus*

tatus to isolated secretions of *Pasimachus subsulcatus* to determine the effectiveness of the secretion and its constituents as a skink predator deterrent. Specifically, we test the following hypotheses: (1) *Pasimachus subsulcatus* is an available prey for *Eumeces inexpectatus*. (2) *Eumeces inexpectatus* refuse to ingest *P. subsulcatus* and other arthropods of similar size and habits under controlled field conditions. (3) Museum specimens of *Eumeces* contain no *Pasimachus subsulcatus* in their digestive tract and no other arthropods of comparable size and habits. (4) *Pasimachus* secretion and individual secretion constituents have no effect on the movement behavior of *E. inexpectatus*.

METHODS AND MATERIALS

Study Area. Field work was conducted in the sandhill portion of the University of South Florida Ecological Research Area. For a complete description of the habitat see Mushinsky (1985).

Availability of Prey. The daily activity cycles of *Pasimachus subsulcatus* and *Eumeces inexpectatus* are asynchronous. *Pasimachus subsulcatus* is nocturnal and active throughout the year, while *E. inexpectatus* is diurnal and active primarily only in the warmer months from April through October. *Eumeces* forage in leaf litter (Fitch, 1954; Vitt and Cooper, 1986). It was important to determine whether or not the beetle occurred in the litter during the day and, therefore, was available to the lizards.

Leaf litter core samples (area of core base = 78.54 cm) were taken randomly from the study area ($N = 150$). Litter depth was determined by measuring the distance from the soil-litter interface to the top of the litter layer. Samples were stored separately, returned to the laboratory, and weighed. They were dried at 70°C for 24 hr, and the dry weight was recorded. Density of each sample was calculated using the formula $D = m/v$, where m is the dry weight (mg) and v is the volume (cm³). The following variables were then estimated: (1) percent contribution to leaf litter of dominant tree species, ranked from most to least frequently monotypic leaf litter type, using the first 50 samples; (2) range of litter depth for each common monotypic leaf litter type; and (3) range of litter density for each common monotypic leaf litter type.

Manipulations were performed in the field to estimate the location of *Pasimachus subsulcatus* during its daytime inactivity period. Five 40-liter glass aquaria were buried in randomly selected locations, with the upper 5 cm above ground. The aquaria were filled to ground level with local soil. In May and June 1986, the months of peak activity for *Eumeces* in west central Florida, individual *P. subsulcatus* were placed in each aquarium with each common litter type (*Quercus geminata*/*Q. virginiana* combined, *Q. laevis*, and *Pinus palustris*). *Quercus geminata* and *Q. virginiana*, and *Quercus laurifolia* and *Q.*

incana (least common species) were combined because these couplets of oaks frequently hybridize and because they share similar leaf morphologies. Low, medium, and high densities of each common litter species were determined by using the minimum, median, and maximum values obtained from field samples. Litter samples were resaturated with water to the moisture level measured prior to the manipulation. Aquaria were covered with wire screens to prevent the beetles from escaping and to reduce predation. After 24 hr, the litter was removed carefully and the position of the beetle was recorded. Each beetle was checked for presence or absence of secretion by squeezing the abdomen and pulling an appendage, thereby simulating a predator attack. Pearson product moment correlations (Sokal and Rohlf, 1981) were calculated for beetle location with both litter depth and litter density.

Feeding Trials. Field feeding trials were conducted to determine if *Pasimachus subsulcatus* are ingested by *Eumeces*. A 1-m² quadrat was selected randomly and enclosed with tin drift fence material to form a test pen. All plant material was removed from the quadrat to facilitate unobstructed observation. Lizards were offered centipedes, millipedes, spiders, orthopterans, and several species of coleopterans including *Pasimachus*. Each individual prey was placed separately in the test pen with a lizard and observed for 1 hr or until the prey was ingested.

Analysis of Gut Contents. The 35 *Eumeces inexpectatus* in the University of South Florida herpetological collection were dissected to check for presence or absence of *Pasimachus subsulcatus* and to determine if prey of similar size and habits were ingested. Museum specimens were collected from a variety of locations in west central Florida. All partially digested coleopterans were identified by elytral fragments. Prey in stomachs and intestines were identified to order. Gut contents were dried at 70°C for 24 hr before weighing.

Response to Isolated Pygidial Secretions. *Eumeces* were housed individually in 2-liter plastic containers and maintained at 28°C on a 12:12 hr light-dark cycle. Lizards were fed *Tenebrio molitor* larvae to satiety weekly and tested one day prior to the scheduled experimental feeding to ensure a similar degree of hunger. The antipredator effectiveness of *Pasimachus subsulcatus* pygidial secretions was evaluated by measuring the movement response of *Eumeces* to commercially synthesized secretion constituents obtained from Eastman Kodak Corporation (Table 1). *Pasimachus subsulcatus* pygidial gland reservoirs were removed from freezer-killed animals and placed in separate ampules. Thomas Eisner and Brad Davidson of Cornell University, Ithaca, New York, kindly identified the secretion constituents. Two individual purified constituents and a mixture of three purified constituents (Table 2) combined in their natural proportions (Table 1) were presented to *Eumeces* in a rectangular Plexiglas test chamber (19 × 21 × 5 cm). The test chamber was partitioned equally into three compartments. Doorways (4 cm²) were cut in the center of the par-

TABLE 1. CHEMICAL CONSTITUENTS OF *Pasimachus subsulcatus* DEFENSIVE SECRETION

Constituent	Formula	Molecular weight	Contribution ^a
Methacrylic acid	$\text{CH}_2=\text{C}(\text{CH}_3)\text{CO}_2\text{H}$	86.09	68.5 ± 7.0
Tiglic acid (<i>trans</i>)	$\text{CH}_3\text{CH}=\text{C}(\text{CH}_3)\text{CO}_2\text{H}$	100.12	25.1 ± 4.6
Angelic acid (<i>cis</i>) ^b	$\text{CH}_3\text{CH}=\text{C}(\text{CH}_3)\text{CO}_2\text{H}$	100.12	4.6 ± 3.0
Isobutyric acid	$(\text{CH}_3)_2\text{CHCO}_2\text{H}$	88.11	0.9 ± 0.4
Senecioic acid ^b	$(\text{CH}_3)_2\text{C}=\text{CHCO}_2\text{H}$	100.12	0.3 ± 0.3
Other			0.6 ± 0.3

^a Mean percentage and SD.

^b Senecioic and angelic acid were not available commercially.

TABLE 2. RESPONSES OF *Eumeces* TO *Pasimachus* PYGIDIAL SECRETIONS^a

	Percent of trials a lizard was in chamber			G value
	A	B	C	
Control: distilled water				
	29.0	41.0	30.0	1.4 NS
Secretion A: Isobutyric acid				
	2.0	61.0	37.0	73.14**
Secretion B: Methacrylic acid				
	2.0	58.0	40.0	61.42**
Secretion C: Tiglic, methacrylic, and isobutyric acid				
	5.0	64.0	31.0	49.11**

^a Each response was compared to the expected random distribution NS = not significant $P \geq 0.05$. Chamber A was always the secretion-labeled chamber. Values were pooled across lizards.

** $P < 0.01$.

titions to allow the lizards access to each compartment (Figure 1). The test room was dark except for lamps illuminating the four sides of the test chamber (Vitt and Cooper, 1986). This technique allowed us to observe the lizard without disturbing it. A lizard was placed in the center chamber and denied access to the other chambers by blocking the doorways with Plexiglas doors for 1 min. Removal of the doors allowed the lizard to move freely between chambers for 10 min. Lizards came to rest in one of the chambers after approximately 10

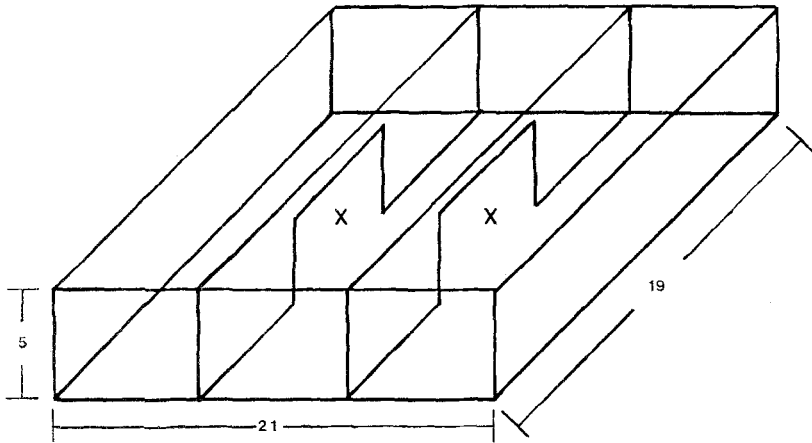


FIG. 1. Test chamber used in isolated secretion experiments. X indicates location of doorways.

min. Fifteen trials per lizard were conducted without stimuli on 10 lizards to determine if the distribution differed significantly from random (0.33 probability of being located in any chamber). Lizards were treated as replicates with multiple trials per lizard. Values were pooled if individual lizard's responses did not differ significantly from the expected random distribution.

Lizards then were tested by placing a paper toweling strip (4×1 cm) across the floor at the entrance to the left and right chambers. A trial consisted of wetting one strip with 0.5 ml of a beetle secretion constituent (experiment) while the other strip was saturated with 0.5 ml of distilled water (control). Experimental and control strips were assigned randomly to the chambers. A lizard was placed in the center chamber and tested in the same manner as the trials without stimuli. After 10 min, the lizard's location was recorded and the trial terminated. A total of 283 trials were conducted, 106 for secretion A (isobutyric acid), 96 for secretion B (methacrylic acid), and 81 for secretion C (tiglic, methacrylic, and isobutyric acids combined). Since the trials for each lizard differed significantly from the expected random distribution, the results for all lizards were combined. The pooled results of each stimulus were tested with a *G*-test goodness-of-fit analysis adjusted for chi-square approximation with Williams' correction formula (Sokal and Rohlf, 1981) to determine if the observed distribution of lizard locations was different from expected.

RESULTS

Litter Measurements and Availability of Prey. Quercus geminata/Q. virginiana ranked first in relative abundance of litter types; *Quercus laevis* was

second, *Pinus palustris* third, and *Q. laurifolia/Q. incana* fourth (Table 3). Low, medium, and high litter depths and densities are listed in Table 4.

In 29 of 40 litter density manipulations (73%), beetles were found at or above the soil-litter interface. Nine beetles (23%) were found in the soil (two beetles died during the experiment). Given a choice, most beetles rest in or just below the litter during their inactive period. Six of the 29 beetles were located at the soil-litter interface while the remaining 23 were above it. Beetles burrowed in the soil most frequently at low litter densities. Seven of the nine beetles that had burrowed into the soil were located only 0.5 cm below the interface. No beetle burrowed more than 3.0 cm into the soil. Beetle location did not correlate with litter depth ($r = 0.34$, $P > 0.05$). Beetle burrowing depths in all manipulated litter types and densities are listed in Table 5.

Eight of nine beetles that burrowed into the soil failed to produce pygidial secretion upon simulation of a predator attack. We assumed that the gland had been depleted prior to the experiment. Only two of 23 beetles located in the litter lacked the secretion, as did one of the six beetles located at the interface. Perhaps beetles that lack secretion tend to burrow into the soil to avoid predators.

Analysis of Gut Contents. Of 68 prey found in the guts of 28 lizards, 58

TABLE 3. RELATIVE ABUNDANCE OF LITTER TYPES

Litter type	Average depth (cm)	No. of samples	Percent of total litter
<i>Quercus geminata/Q. virginiana</i>	3.67	15	37
<i>Q. laevis</i>	2.93	14	34
<i>Pinus palustris</i>	7.50	8	19
<i>Q. laurifolia/Q. incana</i>	2.38	4	10

TABLE 4. LITTER MANIPULATION DENSITIES AND DEPTHS

Litter type	Densities (mg/cm)			Depths (cm)		
	Low	Medium	High	Low	Medium	High
<i>Quercus geminata/</i>						
<i>Q. virginiana</i>	26.0	87.0	148.0	2.0	4.5	7.0
<i>Q. laevis</i>	22.0	70.0	118.0	1.5	3.5	5.5
<i>Pinus palustris</i>	47.0	192.0	337.0	4.5	9.8	15.0

TABLE 5. BEETLE LOCATION IN THREE LITTER TYPES

Litter type	\bar{X} Beetle location ^a (cm)		
	Low	Medium	High
<i>Quercus geminata</i> / <i>Q. virginiana</i>	0.3	0.5	3.5
<i>Q. laevis</i>	-0.9	0.5	4.2
<i>Pinus palustris</i>	-1.7	6.0	^b

^aBeetle location refers to average position in cm ($N = 5$) above or below (+ or -) soil-litter interface.

^bAquaria could not accommodate the high density of *Pinus*.

(85%) were arthropods (Table 6). Seven lizards (20%) contained nothing in the gut. Although coleopterans constituted nearly 15% of the prey, no adult *Pasi-machus subsulcatus* were found; however, three coleopteran larvae were found that could not be identified. Ten items (15%) were not identified. Two intact tenebrionids were found in one lizard. The size of beetles found in the gut was similar to that of *P. subsulcatus*. None of the elytra matched those of *P. subsulcatus*.

Feeding Trials. *Eumeces* readily ingested large lycosid spiders (20 mm maximum body length), adult and larval tenebrionid beetles (two species),

TABLE 6. GUT CONTENTS OF PRESERVED *Eumeces inexpectatus*

Prey type	Percent of lizards with prey type	Percent of total prey
Araneae	33	22.1
Orthoptera	26	16.2
Coleoptera	26	14.7
Unknown	—	14.7
Hymenoptera	26	10.3
Insecta	11	4.4
Lepidoptera	11	4.4
Chilopoda	7	2.9
Insecta (larvae)	7	2.9
Acari	4	1.5
Isopoda	4	1.5
Homoptera	4	1.5
Diptera	4	1.5
Egg	4	1.5

orthopterans, and large centipedes (10 cm in length). *Pasimachus subsulcatus* is well within the size range of these prey. Lycosid spiders placed anywhere in the test pen with *Eumeces* immediately adopted a sustained defense posture, which consisted of rearing up on the posterior legs with the chelicerae poised for attack. The approach of *Eumeces* was met with a series of lunges by the spiders. *Eumeces* subsequently seized the spiders, smashed them against the substrate, and proceeded to dismember and ingest them. *Eumeces* grasped centipedes in their jaws and smashed them against the substrate for up to 1 min. Defensive reactions of the centipedes consisted of multiple bitings and exaggerated writhing movements. Millipedes were attacked but not ingested. No *Pasimachus* were ingested or attacked. Only one lizard tongue-flicked *Pasimachus*. One beetle actually hid under the body of *Eumeces* during the trial without evoking a response from the lizard. Although movement of most prey resulted in an immediate orientation or movement response from *Eumeces*, movement by *Pasimachus* was typically ignored.

Response to Isolated Pygidial Secretions. In trials without stimuli, lizard locations were not significantly different from the expected random distribution (Table 2). Of 283 trials ($N = 11$ lizards), the chamber labeled with the pygidial secretions was occupied in seven (2%) trials. Of the remaining 276 trials, the center chamber was occupied 166 (59%) times and the chamber labeled with the control was occupied 110 (39%) times. The distribution of lizard locations differed significantly from the expected random distribution for all secretions tested (Table 2).

DISCUSSION

Both the vomeronasal and the nasal olfactory systems are important for prey detection by lizards (Simon, 1983). Each system is innervated separately and may be effective at different distances from a stimulus. Our qualitative observations of *Eumeces inexpectatus* support the assertion that tongue-flick rate increases upon presentation of novel yet undesirable prey as well as upon introduction into an unfamiliar aquarium (see De Fazio et al., 1977). Because the specific motivation for tongue flicking may vary (Burghardt, 1973; Cooper and Vitt, 1986a,b, 1987), conclusions from experiments measuring lizard movement in relation to a potential prey may be less ambiguous than those derived from counting tongue flicks.

Although *Pasimachus subsulcatus* is active at night, it is likely that the beetle rests during the day within the foraging microenvironment of the diurnally active *Eumeces inexpectatus*. Examination of *Eumeces* gut contents indicated that the skinks are capable of ingesting prey comparable in habits to *Pasimachus*. Lycosid spiders are nocturnal and rest in litter during the day (per-

sonal observation), yet were found frequently in the guts of *Eumeces*. In fact, most of the prey found in *Eumeces* were nocturnally active and were inactive and hidden during the lizards' foraging period. *Pasimachus subsulcatus* have a thick exoskeleton and may require longer handling times than soft-bodied arthropods of comparable size. Elytral fragments found in the guts of preserved lizards comparable in thickness to those of *Pasimachus* indicate that *Eumeces* are capable of ingesting these hard-bodied arthropods.

Feeding trials confirm that *Eumeces* prey upon relatively large arthropods. Centipedes more than half the length of *Eumeces* were ingested easily. Tenebrionid beetles similar in size, color, and exoskeletal thickness to *Pasimachus* were ingested by *Eumeces*. Large lycosid spiders were killed and ingested within 1 min of a *Eumeces* attack. Orthopteran tibial spines also did not inhibit predation by the lizards. However, millipedes were not ingested. Eisner et al. (1963a) and Smolanoff et al. (1975) have shown that certain millipedes are protected chemically from predation. We conclude that *Pasimachus* is available and within the size and structure range of prey taken by *Eumeces*. The absence of a positive response by *Eumeces* to *Pasimachus* in all feeding trials indicates that lizards either have learned or are programmed genetically to avoid *Pasimachus subsulcatus*.

Investigations regarding chemosensory-mediated prey selection in lizards have been limited to within-clutch analyses of tongue-flick response to nonnoxious prey (Loop and Scovile, 1972; Burghardt, 1973). The chemosensory response of a population of *Eumeces inexpectatus* ($N = 11$) from west central Florida convincingly demonstrates that this lizard typically does not occupy chambers labeled with *Pasimachus subsulcatus* secretion. Lizards walked about the test chamber frequently, and virtually every forward movement was accompanied by a series of tongue flicks. Tongue flicks were directed primarily at the substrate but also at the air immediately ahead of the lizard. Lizards approaching a chamber labeled with a beetle secretion typically halted, reversed direction, and rubbed the snout on the chamber floor for several seconds. After tongue-flicking the *Pasimachus* secretion, lizards often ran to the far chamber. One lizard entered the chamber labeled with a beetle secretion four times. Lizards that entered secretion-labeled chambers did not tongue flick the secretion upon entering the chamber. Once in the secretion-labeled chamber, lizards remained there if they contacted the secretion while attempting to leave. Perhaps these lizards did not detect the secretion upon entering the chamber but may have sensed it while attempting to exit.

Vomer nasal contact with *Pasimachus subsulcatus* pygidial secretion is sufficient to halt the forward progress of *Eumeces inexpectatus*. Eisner (1970) has determined that the elytral sculpturing of certain beetles impedes evaporation of residual pygidial spray. Residual secretion may remain on the elytra for several hours and subsequently still be an effective predator deterrent. Perhaps

predators need only sample these residual molecules, without actually being sprayed, to exhibit an avoidance response. Because *Eumeces inexpectatus* relies heavily on chemoreception to detect their diurnally inactive prey, it may be more economical energetically to use the same sensory mechanisms to avoid concealed noxious prey.

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AGGREGATION PHEROMONE CHARACTERIZATION AND COMPARISON IN *Drosophila ananassae* and *Drosophila bipectinata*

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Abstract—(*Z*)-11-Octadecenyl acetate (Z11-18:Ac) and (*Z*)-11-eicosenyl acetate (Z11-20:Ac) were identified as the aggregation pheromones of *Drosophila ananassae*, and Z11-20:Ac was identified as the aggregation pheromone of *Drosophila bipectinata*. Z11-18:Ac and Z11-20:Ac were not attractive alone; however, in combination with fermenting food odors, the acetates attracted flies of both sexes in a wind-tunnel olfactometer. The pheromones were present in the ejaculatory bulb of sexually mature male flies and transferred to the female during mating. Male *D. bipectinata* released little if any Z11-20:Ac to the food; however, recently mated females released Z11-20:Ac to the surrounding surfaces in just a few hours after mating. *D. ananassae* males, on the other hand, appeared to release more Z11-18:Ac and Z11-20:Ac to the surroundings than mated females. Although *D. bipectinata* males had no Z11-18:Ac, flies were as attracted to Z11-18:Ac as to an equal quantity of Z11-20:Ac. *D. ananassae* were attracted to Z11-18:Ac but not to Z11-16:Ac or Z11-20:Ac. However, Z11-20:Ac in combination with Z11-18:Ac was significantly more attractive than Z11-18:Ac alone.

Key Words—*Drosophila ananassae*, *Drosophila bipectinata*, Diptera, Drosophilidae, aggregation pheromone, (*Z*)-11-octadecenyl acetate, (*Z*)-11-eicosenyl acetate, *cis*-vaccenyl acetate.

INTRODUCTION

In all *Drosophila* species studied to date, aggregation pheromones exist that are produced exclusively by the sexually mature males, attractive to both sexes,

and most active in combination with food odors (Bartelt and Jackson, 1984; Bartelt et al., 1985a,b, 1986, 1988; Moats et al., 1987; Schaner et al., 1987, 1989) In the *virilis* species group, the aggregation pheromones are a blend of hydrocarbons and esters of tiglic and hexanoic acids (Bartelt and Jackson, 1984; Bartelt et al., 1985a, 1986, 1988). The hydrocarbons are transferred by the male to the food media (unpublished). In *D. hydei*, a member of the *repleta* group, the aggregation pheromone is a mixture of ketones along with the esters of tiglic acid (Moats et al., 1987). The esters of tiglic acid are a component of the pheromone blend common to both groups, yet each required compounds with different chemical functional groups in combination with the esters for optimal aggregation response. In the *melanogaster* subgroup of the *melanogaster* group, *D. melanogaster* and *D. simulans* sexually mature males produce (*Z*)-11-octadecenyl acetate (*Z*11-18:Ac) (Bartelt et al., 1985b; Schaner et al., 1987); however, in the *ananassae* subgroup, *D. malerkotliana* males produce (*Z*)-11-eicosenyl acetate (Schaner et al., 1989). In both subgroups the pheromone is stored in the ejaculatory bulb, transferred to the females during mating, emitted by the female to the food within a few hours after mating, and functions as the aggregation pheromone.

We report here the identification, transfer, and bioassay characteristics of the aggregation pheromones in *D. ananassae* and *D. bipectinata*, also of the *ananassae* subgroup.

METHODS AND MATERIALS

Flies. *D. ananassae* (strain 14024-0371) and *D. bipectinata* (strain 14024-0381) were obtained from the National Drosophila Species Resource Center at Bowling Green, Ohio. Flies were reared on Instant Drosophila Medium Formula 4-24 (Carolina Biological Supply Co., Burlington, North Carolina) in 1-liter jars or in 3.5×10 -cm vials under a light-dark cycle of 16:8 hr and 22-24°C.

The method of extraction, chromatography, identification, bioassay, and pheromone transfer have been reported previously (Bartelt and Jackson, 1984; Bartelt et al., 1985b; Schaner et al., 1987, 1989). Briefly, flies were separated by sex at 0-6 hr old and extracted at 6-7 days of age by soaking the flies in hexane at room temperature for 24 hr. Hexane extracts were fractionated on open columns of silicic acid eluted with: hexane; 10% ether in hexane; 50% ether in hexane; and 10% methanol in methylene chloride.

Bioassays were conducted in a wind-tunnel olfactometer containing ca. 1000 (0- to 2-day-old) flies that had been without food or water for approximately 2 hr. An extract, fraction, synthetic compound, or control solvent was applied to a filter paper inserted around the lip of a glass vial. Each bioassay

test consisted of placing two differently treated vials to be compared into the olfactometer for 3 min. The bioassay data were transformed to the $\log(X + 1)$ scale before analysis to stabilize variance, and analysis was done by the method of Yates (1940).

Production, Transfer, and Dispersal of Pheromone. The methods have previously been described (Schaner et al., 1989). Briefly, ejaculatory bulbs were removed from adult male flies at various ages, extracted with hexane, and analyzed by gas chromatography (GC). The transfer of pheromone during mating was quantitated by removing the reproductive tract of females and the ejaculatory bulb and ducts of males immediately after mating, crushing the organs with the head of a dissecting pin, extracting with hexane, and analyzing by GC relative to an internal standard.

RESULTS AND DISCUSSION

Identification of Pheromone Components. Crude hexane extracts of either male or female flies were not active in aggregation assays (2.6 and 2.1 mean catch, respectively, for *D. ananassae* and *D. bipectinata* male extracts alone). Other *Drosophila* frequently require food or food odors in combination with the pheromone to demonstrate or enhance aggregation activity (Bartelt et al., 1985b, 1986, 1988; Schaner et al., 1989). Both *D. bipectinata* and *D. ananassae* crude hexane extract of males were synergistic with fermenting food (Table 1, A). For convenience, solvents such as acetone (Bartelt et al., 1985b; Schaner et al., 1989) and 0.1% acetic acid were tested to mimic the coattraction observed with fermenting food (Table 1, B). Both acetone and acetic acid were effective coattractants. Acetic acid in combination with the crude extracts attracted more flies, so it was used throughout this investigation.

The crude hexane extracts of mature male flies along with acetic acid were clearly active in bioassay for both species (Table 2), whereas the extracts from female flies were inactive. After chromatography of the extracts of male flies, the most active fraction in both species was the 10% ether-hexane fraction, which contains the acetate esters (Bartelt et al., 1985b; Schaner et al., 1987, 1989). Gas chromatography and gas chromatography-mass spectrometry (GC-MS) of the 10% ether-hexane fraction revealed that in *D. ananassae* the fraction was 29% (Z)-11-octadecenyl acetate (Z11-18:Ac) and although no (Z)-11-eicosenyl acetate (Z11-20:Ac) was detected in this fraction, a small amount of Z11-20:Ac was later discovered in the ejaculatory bulb. In *D. bipectinata* the fraction was 50% Z11-20:Ac with no detectable Z11-18:Ac. The position of the unsaturation was determined by GC-MS of the dimethyl disulfide derivative (Nichols et al., 1986).

In bioassay, the 10% ether-hexane fraction accounted for more than all of

TABLE 1. FIVE SERIES OF BIOASSAY EXPERIMENTS WITH EXTRACTS OF *D. ananassae* AND *D. bipectinata* TO ISOLATE AND CHARACTERIZE AGGREGATION PHEROMONE.

Treatment ^a	Mean bioassay catch	
	<i>D. ananassae</i>	<i>D. bipectinata</i>
A. Synergism of crude extracts of males with fermenting food	(N = 12)	
Control	0.4 A ^b	1.6 A
Crude extract of males	2.6 B	2.1 A
Food ^c	10.3 C	10.3 B
Crude extract + food	27.8 D	20.3 C
B. Synergism of crude extract of males with food odors	(N = 12)	
Crude extract of males	2.5 A	2.2 A
Crude extract + acetone	7.4 B	6.0 B
Crude extract + acetic acid ^d	11.4 C	34.9 C
C. Synergism of synthetic pheromone with fermenting food	(N ≥ 12)	
Z11-18:Ac	0.9 B	ND ^e
Z11-20:Ac	ND	0.7 A
Food	6.4 C	19.5 B
Z11-18:Ac + food	24.5 D	ND
Z11-20:Ac + food	ND	57.3 C
Hexane control	0.0 A	0.6 A
D. Comparative bioassay response to synthetic acetates	(N = 18)	
Control (acetic acid)	1.7 A	1.2 A
Z11-16:Ac + acetic acid	2.4 A	1.9 A
Z11-18:Ac + acetic acid	11.3 B	11.4 B
Z11-20:Ac + acetic acid	3.0 A	9.2 B
E. Synergism of synthetic Z11-18:Ac and Z11-20:Ac in <i>D. ananassae</i>	(N = 12)	
Control (acetic acid)	6.9 A	ND
Z11-18:Ac + acetic acid	16.4 B	ND
Z11-20:Ac + acetic acid	6.7 A	ND
Z11-18:Ac + Z11-20:Ac + acetic acid	24.6 C	ND

^aAll fly-derived fractions and their synthetic counterparts were used at 300 ng acetate ester for *D. bipectinata* and 12 ng acetate ester for *D. ananassae*.

^bMeans followed by the same letter were not significantly different at the 5% level (LSD).

^cFermenting food was Formula 4-24 Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, North Carolina) to which active yeast had been added at least 24 hr prior to testing.

^dAcetic acid is a 0.1% solution.

^eNot determined.

TABLE 2. AGGREGATION RESPONSE IN A WIND-TUNNEL OLFACTOMETER TO HEXANE EXTRACTS OF *D. ananassae* AND *D. bipectinata* AND FRACTIONS OF THESE EXTRACTS.

Fraction or extract from respective species	Relative response ^a (N = 8)	
	<i>D. ananassae</i>	<i>D. bipectinata</i>
Crude extract of males	100*** ^b	100***
Hexane fraction	-2 (2.1, 1.9, 10.5) ^c	-8 (3.4, 2.5, 14.3)
10% ether fraction	142*** (1.2, 22.4, 16.1)	224*** (2.4, 11.6, 6.5)
50% ether fraction	44*** (1.4, 6.5, 12.9)	-51 (3.5, 0.8, 8.8)
10% MEOH/CH ₂ Cl ₂ fxn.	9** (1.2, 2.9, 19.5)	-23 (2.7, 1.0, 10.1)
Crude extract of females	7 (1.9, 2.9, 16.6)	-16 (2.6, 1.5, 9.6)

^aRelative response = [(fraction - control)/(male crude extract - control)] × 100.

^b** and *** denote significance of *t* tests vs. controls at the 0.01 and 0.001 level, respectively.

^cThe numbers in parenthesis are mean catch of flies to 0.1% acetic acid control, fraction + 0.1% acetic acid, male crude extract + 0.1% acetic acid.

the activity of the male crude extract (Tables 2 and 3). This suggests that the active compounds were in the 10% ether-hexane fraction, but the crude extract may have had compounds that diminish the aggregation activity. Synthetic Z11-18:Ac and Z11-20:Ac accounted for all the aggregation activity of the male 10% ether-hexane fraction for *D. ananassae* and *D. bipectinata*, respectively.

Since the crude hexane extracts of males were synergistic with fermented food, synthetic Z11-18:Ac and Z11-20:Ac were bioassayed with *D. ananassae* and *D. bipectinata*, respectively, to determine whether they also were synergistic with the aggregation activity of food. The synthetic acetate esters were synergistic with fermenting food (Table 1, C).

Aggregation Activity Specificity. The specificity of the flies' response to a certain chain length Z11-acetate was tested, and *D. ananassae* showed high specificity for Z11-18:Ac and no attraction toward Z11-20:Ac (Table 1, D). Because males possess both Z11-18:Ac and Z11-20:Ac, the two were combined to test for synergism. Z11-18:Ac and Z11-20:Ac together were significantly more attractive than Z11-18:Ac alone (Table 1, E). *D. bipectinata* responded equally to either Z11-18:Ac or Z11-20:Ac even though males produce only Z11-20:Ac. Neither species was attracted to Z11-16:Ac. In previous studies (Schaner et al., 1989), *D. melanogaster* and *D. simulans* were

TABLE 3. COMPARATIVE AGGREGATION ACTIVITY OF EXTRACTS, FRACTIONS, AND SYNTHETIC COMPOUNDS

Treatment	Mean bioassay catch (N = 8)	
	<i>D. ananassae</i>	<i>D. bipectinata</i>
Control (acetic acid) ^a	2.2 A ^b	5.8 A
Male crude + acetic acid	10.0 B	16.7 B
-10% Ether fxn. + acetic acid	15.6 C	23.7 C
Syn. Z11-18:Ac ^c + acetic acid	14.7 BC	ND ^e
Syn. Z11-20:Ac ^d + acetic acid	ND ^e	25.1 C

^a Acetic acid is a 0.1% solution.

^b Means followed by the same letter were not significantly different at the 5% level (LSD).

^c 12 ng matching contents in 10% ether fxn. and crude from *D. ananassae* males.

^d 160 ng matching content in 10% ether fxn. and crude from *D. bipectinata* males.

^e not determined.

attracted only to Z11-18:Ac, but *D. malerkotliana* responded to both Z11-18:Ac and Z11-20:Ac. *D. melanogaster* preferred Z11-16:Ac slightly over controls, but neither of the other species were attracted to Z11-16:Ac. Of the five species so far tested against these three acetate esters, there was a range of specificity that does not follow taxonomic lines, but varies within each species group. A more complete rationale for the specificity variation awaits further studies of other species.

Dose Response. For *D. ananassae*, when a dose of Z11-18:Ac was increased from 1.2 ng to 12 ng, there was a dramatic increase in the aggregation activity (Figure 1). Activity increased only slightly when the dose increased to 1200 ng. The dose of 12 ng of Z11-18:Ac was the lowest dosage to result in a maximum dose plateau so far observed for this type of aggregation pheromone. For *D. bipectinata* (Figure 1), the same trend was observed. There was also a dramatic increase in response when the dose of Z11-20:Ac was increased from 20 ng to 200 ng. From 200 ng to 2000 ng, and from 2000 ng to 20,000 ng, the response of the flies increased slightly, but not significantly.

Location, Production, Transfer, and Dispersal of Aggregation Pheromones. As previously observed in *D. melanogaster*, *D. simulans*, and *D. malerkotliana* (Brieger and Butterworth, 1970; Schaner et al., 1987, 1989), the ejaculatory bulb of *D. ananassae* and *D. bipectinata* mature male flies was the site of storage of the aggregation pheromone. Analysis of *D. ananassae* ejaculatory bulb contents (Figure 2) showed an increase in Z11-18:Ac with age from 0 ng/fly at day 1 up to a plateau of ca. 52 ng/fly on day 7. The ejaculatory bulbs of male *D. ananassae* also contained Z11-20:Ac, which increased after day 1 to a plateau of about 15-17 ng/fly. *D. ananassae* was the only species

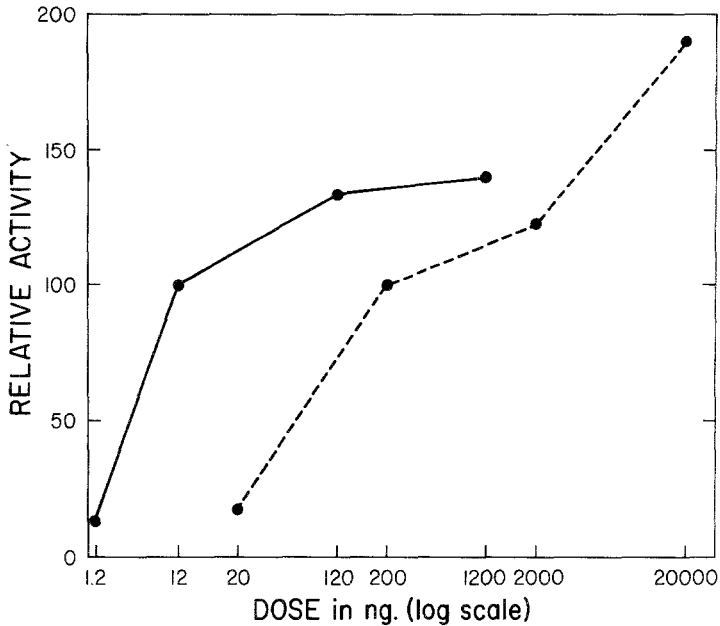


FIG. 1. Dose-response curves relative to the amount of pheromone that was hexane extractable from content of one mature male fly (100%). Solid line was the response for *D. ananassae* to Z11-18:Ac. Dashed line was the response for *D. bipectinata* to Z11-20:Ac.

found to possess both the aggregation pheromone common to the *melanogaster* subgroup (Z11-18:Ac) and that of the *ananassae* subgroup (Z11-20:Ac).

D. bipectinata male flies produce ca. 30 ng of Z11-20:Ac at 1 day of age (Figure 2) and ca. 300 ng/fly by 4 days of age, where they reach a plateau. Z11-18:Ac was not detected in ejaculatory bulb extracts of *D. bipectinata*, even though they responded to Z11-18:Ac in aggregation assays (Table 1, D). The amount of Z11-20:Ac produced in the ejaculatory bulb and the bioassay response towards both Z11-18:Ac and Z11-20:Ac was the same as that observed for *D. malerkotliana* (Schaner et al., 1989).

As was observed in *D. melanogaster* (Brieger and Butterworth, 1970; Butterworth, 1969; Jallon et al., 1981; Bartelt et al., 1985b), *D. simulans* (Schaner et al., 1987), and *D. malerkotliana* (Schaner et al., 1989), a portion of the aggregation pheromone in the ejaculatory bulb of *D. ananassae* and *D. bipectinata* was transferred to the female fly during mating (Table 4). *D. bipectinata* male flies transferred about two thirds of their Z11-20:Ac to the female during mating and nearly half of the Z11-20:Ac was released by the female flies to the vial within 6 hr. Neither mated nor virgin males of *D. bipectinata* released

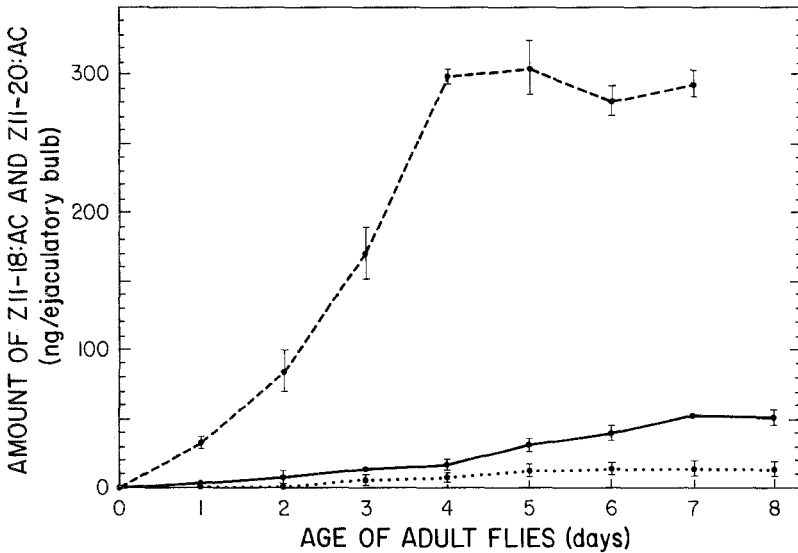


FIG. 2. Pheromone content in the ejaculatory bulb of male flies as they age. Dashed line for Z11-20:Ac in *D. bipectinata*. Solid line for Z11-18:Ac and dotted line for Z11-20:Ac in *D. ananassae*. The bars indicate standard deviation.

Z11-20:Ac to their surroundings in 6 hr. *D. ananassae* male flies, on the other hand, transferred both Z11-18:Ac (ca. 14 ng/fly) and Z11-20:Ac (ca. 5 ng/fly) to the female reproductive tract. Within 6 hr after mating, female flies released only a trace of both compounds to the vial and had retained most of the transferred compounds in their reproductive tracts. Both mated and virgin *D. ananassae* males release Z11-18:Ac to their surroundings in 6 hr (Table 4). This was the first time that the release by males, either mated or virgin, was greater than the release by mated female flies. By 12 hr after mating, the mated females had released approximately half of the Z11-18:Ac that was transferred. The release by mated males during 12 hr after mating was ca. 35 ng of Z11-18:Ac and ca. 2 ng of Z11-20:Ac per fly, which was about seven times more than was released by the mated females. Virgin males also released similar amounts. This suggests that there was not only release, but also synthesis of at least Z11-18:Ac. Unlike the *Drosophila* species observed so far, *D. ananassae* males were the primary releasers of the aggregation pheromone rather than mated females, as observed here for *D. bipectinata* and previously for *D. melanogaster* (Bartelt et al., 1985b), *D. simulans* (Schaner et al., 1987), and *D. malerkotliana* (Schaner et al., 1989).

In nature, mating, feeding, and oviposition all occur at the same location, and flies are attracted to these locations by odors (Spieth, 1974). We believe this attraction is caused not only by food odors but often by a combination of

TABLE 4. TRANSFER OF AGGREGATION PHEROMONES FROM MALES TO FEMALES DURING MATING AND RELEASE OF PHEROMONES BY MATED MALES AND FEMALES

Source ^a	Pheromone (ng/fly)		
	<i>D. ananassae</i> ^b		<i>D. bipectinata</i> ^c
	Z11-18:Ac	Z11-20:Ac	Z11-20:Ac
Virgin male	56	18	297
Mated male	15	6	110
Virgin female	0	0	0
Mated female	14	5	200
6 hrs (12 hr) after mating ^d			
Mated male	19 (21)	8 (6)	117
Vial	6 (35)	0 (2)	0
Mated female	9 (7)	4 (0)	126
Vial	tr (5)	tr (tr)	96
Virgin male	47 (45)	16 (16)	300
Vial	8 (38)	0 (3)	0

^aEjaculatory bulb for males and reproductive tract for females.

^b $N \geq 2$ groups of 10.

^c $N \geq 3$ groups of 5.

^dFlies were stored in vials for 6 hr after mating and then the ejaculatory bulb for males and reproductive tract for females were extracted. The vials were also extracted. The remainder of the fly was extracted but the content of acetates was always below detectable levels.

food odors and fly-derived compounds. *D. ananassae* and *D. bipectinata* males produce an aggregation pheromone that is synergistic with food odors. In the case of *D. bipectinata*, males cannot release the pheromone, but a recently mated female is capable of emitting levels of Z11-20:Ac that are attractive with a food source in our olfactometer. On the other hand, a *D. ananassae* male or recently mated female can release levels of Z11-18:Ac that are also attractive with a food source. These results suggest that in nature, flies are not only attracted to feeding sites by food odors, but they may demonstrate an even stronger attraction to food sources that are already occupied by others of the same species.

In summary, aggregation pheromones in *D. ananassae* and *D. bipectinata* have been identified and characterized, and attraction towards them has been demonstrated. The pheromones are (Z)-11-octadecenyl acetate and (Z)-11-eicosenyl acetate in *D. ananassae* and (Z)-11-eicosenyl acetate in *D. bipectinata*. In both species the pheromones are produced exclusively by sexually mature male flies, stored in the ejaculatory bulb, transferred to the female during mating, synergistic with food odors, and attracted flies of both sexes in a wind-tunnel olfactometer.

The *ananassae* species group is divided into two species complexes based on the structure of the male genitalia (reviewed by Lemeunier et al., 1986). *D. malerkotliana* and *D. bipectinata* of the *bipectinata* complex both produce Z11-20:Ac, which is released by mated female flies, and they respond to either Z11-18:Ac or Z11-20:Ac. *D. ananassae* in the *ananassae* complex produces both Z11-18:Ac and Z11-20:Ac, which is released by mature male flies, and they respond to Z11-18:Ac and a combination of Z11-18:Ac with Z11-20:Ac, but not to Z11-20:Ac alone. Whether the production and response specificity is present in other members of the *ananassae* and *bipectinata* complexes remains to be determined.

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1,5,7-TRIOXASPIRO[5.5]UNDECANE, A PHEROMONE ANALOG WITH HIGH BIOLOGICAL ACTIVITY FOR THE OLIVE FRUIT FLY, *Dacus oleae*

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Abstract—1,5,7-Trioxaspiro[5.5]undecane, an analog of the major sex pheromone (olean) of the olive fruit fly, was synthesized via two different routes and tested for biological activity under laboratory and field conditions. In laboratory tests, its activity was comparable to that of olean, especially when a stabilizer or a high concentration was used. In field tests, its activity reached the level of olean only when a stabilizer and an open-type dispenser, which allows high rates of evaporation, were used. The residual activity of the analog dispensers did not exceed two weeks both under laboratory and field conditions compared to over four months for olean.

Key Words—Olive fruit fly, *Dacus oleae*, Diptera, Tephritidae, pheromones, attractants, sex attractants, pheromone analog, 1,5,7-trioxaspiro[5.5]undecane.

INTRODUCTION

The olive fruit fly, *Dacus oleae* (Gmelin), is a major pest of olives in the Mediterranean region. Present control practices involve the use of wide-spectrum insecticides with serious ecological and toxicological consequences. Scientists in all interested countries are working on the development of alternative control methods. The finding of a four-component female pheromone blend for this pest (Baker et al., 1980; Mazomenos and Haniotakis, 1981, 1985) has stimu-

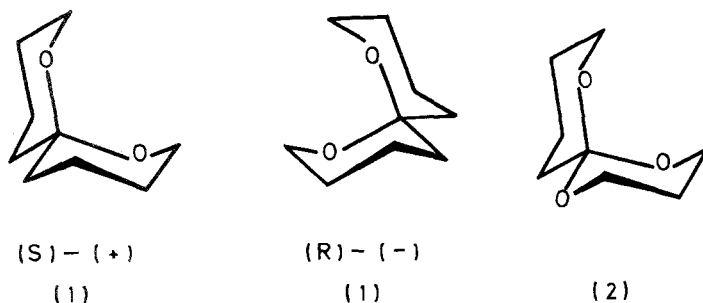


FIG. 1. Chemical structures of 1,7-dioxaspiro[5.5]undecane, the major pheromone of *Dacus oleae* (1), and its analog, 1,5,7-trioxaspiro[5.5]undecane (2).

lated research towards its use for the development of such methods (Haniotakis et al., 1986b, 1987; Montiel and Mata, 1984). The spiroacetal, 1,7-dioxaspiro[5.5]undecane (olean), (1 in Figure 1) is the major component of the blend, which acts as a potent, long-range male attractant and which is released as a racemate (Haniotakis et al., 1986a). The same compound was also found in male flies (Mazomenos and Pomonis, 1983; Haniotakis et al., 1986a), which is as attractive to males as olean isolated from females. No female attraction has been observed, however, to olean of either male or female origin. EAG studies of the racemic mixture of synthetic olean showed similar response of male and female antennae (Van der Pers et al., 1984). Olean isolated from males appears to be also racemate but verification of first results is still required. Further studies showed that (*R*)-1 (Figure 1) is the long-range attractant for males while (*S*)-1 (Figure 1) is an arrestant and aphrodisiac for females (Haniotakis et al., 1986a). We synthesized analogs of olean to find cheaply available attractants (or repellents) and for basic studies on insect olfaction. The synthesis of 1,5,7-trioxaspiro[5.5]undecane, which in this paper will be called analog for convenience, via two different routes, and its biological activity under laboratory and field conditions are reported here.

METHODS AND MATERIALS

Synthesis of 1,5,7-Trioxaspiro[5.5]undecane (Analog). According to the literature, spirocyclic orthoesters are commonly synthesized from the corresponding lactones either by *p*-toluene sulfonic acid-catalyzed condensation with a diol in refluxing benzene (LeMahieu and Kierstead, 1970; Saucy et al., 1977) or by epoxide treatment in the presence of Lewis acid (Bodenbenner, 1959; Inoue and Kataoka, 1965; Imakure, 1971).

The above methods were proven unsatisfactory in the case of the condensation of the simple 1,5-pentanolide (δ -valerolactone) with propane-1,3-diol.

The synthesis of 1,5,7-trioxaspiro[5.5]undecane was realized by two high-yield reaction schemes:

A. Starting from 5-hydroxypentanitrile (Laronze et al., 1980) (Figure 2) a Pinner reaction (Roger and Neilson, 1961) gave the 2-iminotetrahydropyran hydrochloride (**3**). A mixture of **3** with a slight excess of propane-1,3-diol in anhydrous ether was refluxed for 4 hr and, after a simple work-up, pure 1,5,7-trioxaspiro[5.5]undecane (**2**) was obtained, in 80% overall yield.

B. Starting from 1,5-pentanolide (Figure 3), pure 2,2-diethoxytetrahydropyran (**4**) was easily obtained according to the literature (Deslongchamps et al., 1975). A mixture of 8.7 g (0.05 mol) of 2,2-diethoxytetrahydropyran, 3.8 g (0.05 mol) of propane-1,3-diol and 0.01 g *p*-toluenesulfonic acid was gently heated in a distillation apparatus equipped with a small Vigreux column so that ethanol distilled slowly (80–90°C). When ethanol ceased to distill, 0.1 g of anhydrous sodium carbonate was added and the mixture was distilled under medium vacuum. One main fraction of 1,5,7-trioxaspiro[5.5]undecane (**2**) was obtained at 70–74°C and 8–10 mm Hg (7 g, 88% yield).

The biological tests were made with samples of material obtained after two distillations with the following physical and spectral data: Boiling point, 70°C at 8 mm Hg; purity by GC, more than 95% in a Perkin Elmer 8420 capillary gas chromatograph, fitted with a flame ionization detector and a 30-m SE-30 capillary column. Helium was used as carrier gas and the instrument was programmed at 2°C/min from 100 to 230°C. IR (cm⁻¹) 1200, 1140, 1070, 1030 (film on Perkin Elmer 397 spectrometer). [¹H]NMR(δ): 1.18–1.36 (m, 8H); 3.38–4.01 (m, 6H) (CDCl₃ on 80 MHz, Varian FT-80A Spectrometer). MS (*m/z*): 158(M, 4%), 128(55, 7), 113(12, 5%), 101(37, 5%), 100(100%),

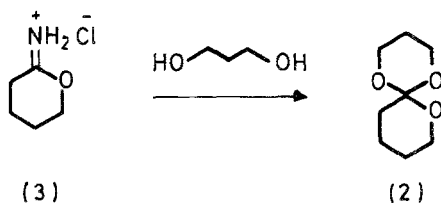


FIG. 2. Synthesis of 1,5,7-trioxaspiro[5.5]undecane (**2**) from 2-iminotetrahydropyran hydrochloride (**3**) and propane-1,3-diol.

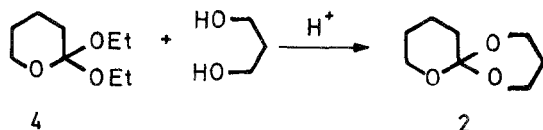


FIG. 3. Synthesis of 1,5,7-trioxaspiro[5.5]undecane from 2,2-diethoxytetrahydropyran (**4**) and propane-1,3-diol.

83(8%), 72(7, 5%), 56(14, 5%), 55(26, 4%), 43(19, 4%), 42(35, 8%), 41(34, 3%). A Hewlett Packard 5970 mass spectrometer was interfaced with a Hewlett Packard 5980 gas chromatograph. The MS were obtained at 70 eV. The separations were accomplished in a 15-m capillary SE-30 column by using helium as carrier gas.)

Laboratory Bioassays. The biological activity of the analog on males was compared to that of racemic olean in an olfactometer, which is described in detail by Haniotakis (1974). It consisted of a wooden $1.38 \times 0.96 \times 0.38$ -m box with a clean Plexiglas top, divided into two sections by a partition along its long axis so that two materials can be tested simultaneously. A regulated air stream inside each section carried the chemical stimulus dispensed from a 1-ml low-density polyethylene vial loaded with 10 or 35 mg of the compounds to be tested. Responding insects entered a glass cage upwind for each of the materials compared, where they were counted. The response of 100 males was tested once per day during the last 4 hr of the photophase, a period that coincides with mating and male response to pheromones (Haniotakis, 1974). The analog was tested with and without stabilizer. The use of a stabilizer was decided upon after the anticipated instability of the analog molecule was verified with TLC (diethylether). When used, the stabilizer was added at a ratio of 33% and was of the following composition (%): (1) benzophenone 23.8; (2) BHT 28.8; (3) quinoline 32.8; (4) triethylamine 14.6. These substances were selected for their antioxidant and UV-absorption properties. Controls were empty vials when no stabilizer was added to the analog or vials containing the same amount of stabilizer which was added to the analog. To reduce high variation of *D. oleae* response to pheromones, which is usually observed in laboratory bioassays, tests were grouped for statistical analysis according to pairs of dispensers compared and insect batch used. For each test, analog and olean dispensers as well as insects used were of the same or very close age. Insect age varied from 4 to 18 days and dispenser age from 0 to 19 days. Lab-cultured flies were used for the tests. One-day-old flies (unmated) were immobilized by chilling at $2-4^{\circ}\text{C}$ for 1-2 hr and separated according to sex. They were kept in 270 cm^3 screen cages under artificial light (3000 lux intensity) and 10:14 hr dark-light regime. Temperature was maintained at $25 \pm 2^{\circ}\text{C}$ and relative humidity at $65 \pm 5\%$.

Field Tests. The biological activity of the analog at the concentration of 25 mg was compared to that of an equal concentration of racemic olean in six sets of field tests, in each of which a different formulation of the analog was used. The formulations tested were: (1) closed white low-density polyethylene vial without stabilizer, (2) closed white low-density polyethylene vial with stabilizer, (3) closed white high-density polyethylene vial with stabilizer, (4) rubber stopper without stabilizer, (5) rubber stopper with stabilizer, and (6) open, black low-density polyethylene vial with stabilizer and activated charcoal. For olean, only closed white low-density polyethylene vials without stabilizer were used. All polyethylene vials were of 1 ml volume. Controls were not used in

field experiments because it was known from previous experiments that unbaited sticky traps of the type used here capture no olive flies. Plywood sticky rectangles measuring 15 × 20 cm were used as traps. When used, stabilizer was of the same composition and at the same ratio as that used in lab tests. Olean and analog dispensers were of the same age except in a few cases (indicated) in which more than one analog formulation was tested at the same time, and the olean dispensers were no more than 30 days old. Olean dispensers of the type used here have an active life of about four months (Mazomenos et al., 1983). One trap was placed in every other olive tree, i.e., at distances that did not exclude trap competition. Traps were checked and cleaned once per week.

RESULTS

Laboratory Bioassays. The biological activity of the analog obtained by either method of synthesis is the same. Table 1 shows the response of *D. oleae* male flies to olean, analog, and to controls.

TABLE 1. RESPONSE OF *D. oleae* MALES TO OLEAN, ITS ORTHOESTER ANALOG, 1,5,7-TRIOXASPIRO[5.5]UNDECANE, AND CONTROLS IN LABORATORY BIOASSAYS—ATHENS 1986

Test group ^a	Number of tests	Percent of male response to			Age (days)		
		Olean	Analog	Control	Insect	Dispenser	
						Olean	Analog
1	4		38.8 (9.8) ^b	24.3a (4.3)	4-7		0-3
2	3		40.7 (9.1)	23.3c (11.2)	4-8		0-2
3	5	34.6 (12.1)	37.4c (11.3)		9-15	0-5	0-5
4	4	58.0 (12.3)	34.3a (6.6)		6-14	11-19	11-19
5	4		81.1 (7.5)	37.1b (11.2)	6-9		5-8
6	3	62.8 (11.9)	40.0c (16.0)		8-10	1-3	0-2
7	8	34.8 (12.2)	33.7c (10.0)		5-17	0-12	0-12

^aIn test groups 1-4 no stabilizer was added; 10 mg of the analog was used. In test groups 5-7 a stabilizer was added; 35 mg, 10 mg, and 35 mg of the analog were used, respectively.

^bValues in parentheses are standard deviations. Pairs of values are *a*, significantly different, Student's *t* test, $P = 0.05$; *b*, significantly different, Student's *t* test, $P = 0.001$; and *c*, not significantly different.

In two (Nos. 1 and 5) of the three groups (Nos. 1, 2, and 5) of tests in which the analog was compared with controls, i.e., nonactive materials, male response to the analog was significantly higher than to the controls. In the third group (No. 2), although not significantly different, probably due to the small number of replicates, the difference in favor of the analog still existed. It should

TABLE 2. TOTAL NUMBERS OF *D. oleae* FLIES CAPTURED ON STICKY BOARDS BAITED WITH 25 mg OF OLEAN OR ITS ANALOG, 1,5,7-TRIOXASPIRO[5.5]UNDECANE^a

Date	Flies captured by				Analog Formula ^b	Dispenser age (days)	
	Olean		Analog			Olean	Analog
	Males	Female	Males	Female			
May 29	1	0	2	1	a	1-7	1-7
June 12	29	1	0	1		8-21	8-21
June 19	84	1	2	0		22-28	22-28
June 26	72	1	1	0		29-35	29-35
July 3	20	0	0	0		36-42	36-42
July 10	21	0	0	0		43-50	43-50
Aug. 28	24	0	7	0	a+	1-7	1-7
Sept. 4	52	0	11	0		8-14	8-14
Sept. 11	112	0	2	0		15-21	15-21
Sept. 18	74	0	0	0		22-28	22-28
Sept. 25	184	0	0	0		29-35	29-35
Sept. 18	195	0	23	0	b+	1-7	1-7
Sept. 25	233	0	4	0		8-14	8-14
Oct. 2	155	2	0	0		15-21	15-21
Oct. 2	155	2	63	0	c	15-21	1-7
Oct. 9	61	0	0	0		22-28	8-14
Oct. 21	87 ^c	0	64 ^c	0	c+	8-12	1-5
Nov. 5	82	3	4	2		13-27	6-12
Oct. 16	17 ^c	0	15 ^c	0	d+	1-7	1-7
Oct. 21	87 ^c	0	64 ^c	0		8-12	8-12
Nov. 5	82	3	14	1		13-27	13-27

^aSix traps from May 25 to July 10, and three traps from August 29 to November 5; Athens, Greece, 1986.

^ba = low-density polyethylene vial, white, closed; a+ = same as above plus stabilizer; b+ = high-density polyethylene vial, white, closed plus stabilizer; c = rubber stopper; c+ = rubber stopper plus stabilizer; d+ = low-density polyethylene vial, black, open plus stabilizer, plus activated charcoal.

^cMale captures not significantly different, Student's *t* test, *P* = 0.05. Means not followed by letter are not significantly different.

be pointed out that in the group of tests in which the analog was used at the concentration of 35 mg (No. 5), the difference was much greater (highly significant) than that in the groups with the concentration of 10 mg (Nos. 1 and 2).

In the four groups of tests in which the analog was compared with olean (Nos. 3, 4, 6, and 7), male response was not significantly different in any except one (No. 4) in which no stabilizer was used and dispenser age was between 11 and 19 days. The addition of stabilizer and the use of a higher concentration increased the activity and prolonged the active life of the analog dispenser (No. 7).

Field Tests. Table 2 shows the numbers of *D. oleae* flies captured on olean and analog traps. Traps baited with analog contained in closed white low-density polyethylene vials without stabilizer captured very few flies whose accidental arrival to the trap cannot be excluded. Addition of stabilizer to the above vials increased the number of male flies caught during the first two weeks, which, however, was significantly lower than that caught on traps baited with olean. Use of high density polyethylene vials with stabilizer reduced trap performance, measured by the ratio of males caught on olean and analog traps. Use of rubber stoppers without stabilizer, a dispenser that allows high evaporation rates, increased trap performance but not to the level of olean traps and only during the first week. Use of rubber stoppers with stabilizer increased trap performance to the level of olean but did not prolong its active life. Use of open black vials with stabilizer and activated charcoal increased trap performance to the level of olean and extended its active life to two weeks.

DISCUSSION

Laboratory and field tests clearly showed that the analog of the major pheromone of *D. oleae* is biologically active and that its activity, expressed in male response in lab tests and numbers of males caught on traps in field tests, can be comparable to that of olean; however, stabilizer, open-type dispensers, or high concentration must be used for the analog. The limited residual activity of analog dispensers, not exceeding two weeks at best, indicates that the stabilizer used does not adequately protect the analog molecule from decomposition. The higher activity of the open-type dispensers, which allow high evaporation rates, and of the dispensers with high concentrations of analog, indicate a lower level of unit activity for the analog. More work is required for effective protection of the analog molecule from decomposition and for accurate regulation of its release rates for improvement of its field performance. If this is achieved, the analog can replace olean in steadily expanding practical applications, contributing to substantial cost reduction.

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POSSIBLE INDUCTION BY ESTROUS COWS OF PHEROMONE PRODUCTION IN PENMATES

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Abstract—The primary attractive odors of estrous cows apparently induced production of secondary attractive odors in penmates. Presence of both odors was reflected by the bull's olfactory behavior. The inducing effect was strongest on the day of estrus and the day before. The information content of the primary and secondary odors was different since a sexually experienced bull was able to distinguish olfactorily between estrous and nonestrous cows. The secondary odors attracted at least transient bull's interest, resulting in sniffing, urine tasting, and flehmen reactions, but not mounting attempts. The induced production of attractive odors and lack of precise smell discrimination in penmates may lead to erroneous detection of estrus in cows and heifers that show false estrous signs without corresponding changes in their reproductive organs. Similar factors may be involved in triggering mounting among feedlot steers (buller steer syndrome).

Key Words—Bovine reproduction, estrus detection, estrous behavior, mounting behavior, estrous odor, flehmen, buller syndrome, reproductive pheromones, pheromone emission, smell sensitivity.

INTRODUCTION

The efficiency of artificial insemination (AI) in cattle is decreased by difficulties with accurate estrus detection because of inadequate observation of animals and/or overestimation of estrous signs. In the first case low personnel to animal ratios and demands for high work productivity do not allow sufficient time for undistracted observation of cow behavior. Occasional observation while performing other chores often fails to detect estrus, especially in postpartum cows

shortly after the resumption of ovarian activity when they frequently show silent heat or vague estrous signs. The estrus detection rate is low, and AI and conception are delayed.

In the second case many detected cows are not in true estrus because their estrous signs are not sufficiently scrutinized and compared with properly kept records. The detection rate is falsely high but the subsequent conception rate is low. In addition, inseminating cows in each reported "heat" increases the cost of AI and animal handling and the danger of uterine infections.

There are abnormal conditions when estrus in cattle occurs in shorter than the normal three-week intervals. Insufficient luteal function in postpartum beef cows shortens their estrous cycles (Odde et al., 1980) and cows with cystic ovarian syndrome may show behavioral signs of estrus quite erratically (Youngquist, 1986). However, there seem to be also physiological mechanisms causing some heifers and cows to show signs of heat without concurrent estrous changes in their internal reproductive organs.

There are indications that females in proestrus and estrus or under hormonal treatment may induce behavioral changes in nonestrous penmates that may reach an intensity sufficiently high to be erroneously reported as heat. This phenomenon has been observed in cattle (Kedrov, 1952; Glód, 1961; Lojda, 1966, 1968), primates (Ruiz de Elvira et al., 1982, 1983), and probably exists in carnivores (Hradecký, 1985). Reports also suggest associated induced metabolic changes, such as production of estrus-related compounds in cagemate foxes (Bailey et al., 1980) and urinary pH changes in closely spaced penmate cows (Hradecký, unpublished data). These inductive changes are likely mediated by airborne odorous signals (pheromones) among animals. While pheromonal effects on reproduction have been extensively studied in laboratory animals (Aron, 1979; Bronson, 1979; Milligan, 1980), similar studies in domestic animals remain in the initial phases (Izard, 1983; Vandenbergh and Izard, 1983).

This paper presents data on likely pheromonal influences of the estrous cows on nonestrous penmates in close proximity that were reflected by increased, although transient, olfactory interest of a teaser bull in the penmates during heat checking. These inductive influences were studied in a small group of animals to allow separation of the mutual influences. Multiple interactions in a larger group would be inseparable at this stage of study due to the lack of more precise measurable parameters.

METHODS AND MATERIALS

Three Holstein cows used for a study of bull-cow interactions (Hradecký et al., 1983) were kept together with a mature Hereford steer in a 250-m² dirt pen. During the nine-month study, the cows were tested daily for signs of heat by a teaser bull in an isolated area. The estrous cows were released from the

pen and tested after the bull had checked the nonestrous cows. The steer was present during the heat checking. Each daily observation lasted about 20 min until the bull lost interest in the cows. Evaluation of the bull's interest and flehmen reactions toward the estrous cows has been reported earlier (Hradecký et al., 1983). For this report the flehmen reactions toward the nonestrous cows and the steer were evaluated.

The bull's flehmen reactions toward each nonestrous cow were tallied separately for the days when some other cow was in heat or one day before heat (days 0 and -1; "induction days") and for the "clear" days when no other cow was in days -1 and 0 of estrus. All data collected from day -3 to +1 around the cow's own estrus were excluded. During this period cows emit estrus-related odors (Hradecký et al., 1983) and the effects of their own heat and induction on cow olfactory attractivity might be confounded.

The data were evaluated with the nonparametric χ^2 test (Daniel, 1978) with the null hypothesis of no difference between the averages for the induction and clear days in each of the three cows.

RESULTS

Interactions between the bull and the steer were dependent on the estrous cycle stages of penmate cows. During the heat checking, the bull was usually indifferent toward the steer but turned aggressive when the steer tried to approach an estrous cow and mount her. However, on repeated occasions the bull was attracted to the steer, which in turn did not show the usual fear and evasion. The bull typically nuzzled the steer's prepuce, stimulated him to urinate, tasted his urine (Figure 1), and showed repeated flehmen reactions (Figure 2). These peculiar periods of the bull's olfactory interest were observed only when a penmate cow was coming into heat but had not yet attracted the bull's full attention, or before an estrous cow was released into the bull's proximity. A few times the bull's interest in the steer was observed one to two days after heat in a penmate cow.

Interactions between the bull and the nonestrous cows showed that on days when some other cow was approaching heat or was in heat (induction days) the remaining two nonestrous penmate cows were also more attractive for the bull than on the clear days when no cow was in heat or close to it. During the induction days, the bull showed increased olfactory interest in the nonestrous cows by sniffing, urine tasting, and flehmen reactions but with no mounting attempts. Table 1 summarizes the data for both the induction and clear days in terms of the bull's flehmen reactions observed (total and average per daily observation) toward each of the three cows and compares them to similar data for the two (-1 and 0) estrous days (Hradecký et al., 1983).

The average frequencies of the bull's flehmen reactions toward each nones-



FIG. 1. The teaser bull (right) nuzzles the steer's prepuce, stimulates him to urinate, and tastes his urine.



FIG. 2. The teaser bull shows flehmen reaction after tasting the steer's urine.

TABLE 1. NUMBERS OF INDUCTION, CLEAR, AND ESTROUS DAYS AND NUMBERS OF FLEHMEN REACTIONS (f = TOTAL, \bar{X} = AVERAGE) OBSERVED FOR EACH COW DURING 9-MONTH STUDY

Day	Cow No.		
	884	885	886
Clear	$N = 163$	$N = 179$	$N = 164$
	$f = 90$	$f = 154$	$f = 92$
	$\bar{X} = 0.55$	$\bar{X} = 0.86$	$\bar{X} = 0.56$
Induction	$N = 33$	$N = 37$	$N = 38$
	$f = 26$	$f = 34$	$f = 36$
	$\bar{X} = 0.79$	$\bar{X} = 0.92$	$\bar{X} = 0.95$
Estrous	$N = 22$	$N = 25$	$N = 23$
	$f = 66$	$f = 64$	$f = 61$
	$\bar{X} = 3.00$	$\bar{X} = 2.56$	$\bar{X} = 2.65$

trous cow on induction and clear days were significantly different ($P < 0.005$) from the overall expected average 0.70 of reactions per observation period. The flehmen frequencies were consistently higher on the induction days than on clear days. There were no significant differences in the ability of individual estrous cows to induce the increased attractivity of nonestrous penmates or in the susceptibility of individual nonestrous penmates to the inducing effects of estrous cows. Also, no differences in the induction susceptibility between the first and the second half of the estrous cycle were noted. However, these differences may become evident in studies with larger cow groups and more precise methods of measurement.

DISCUSSION

Numerous reports have confirmed the existence of airborne chemical signals involved in cattle social communication and reproduction, although there is so far no consistent information about the chemical nature of the odorous compounds involved. Cows in heat emanate estrus-related odors (pheromones) that have been indirectly confirmed by the observations and experiments with bulls (Hart et al., 1946; Donovan, 1967; Sambraus, 1969a,b; Sambraus and Waring, 1975; Paleologou, 1977a,b; Hradecký et al., 1983), trained dogs (Akhlebininskii and Ishutov, 1974; Kiddy et al., 1978, 1984; Kiddy and Mitchell, 1981; Hawk et al., 1984), and rats (Ladewig and Hart, 1981). The odorous compounds are apparently produced in an unknown source in the body of the estrous cow and are distributed throughout her body by the bloodstream. Then they occur in many body fluids and effluents such as urine, vaginal secretions,

milk, skin secretions, saliva, and breath. The sexual behavior of animals indicates that especially the increased secretion of urogenital fluids during estrus provides a rich source of these odorous compounds (Nishimura et al., 1983, 1984; Klemm et al., 1987). The odor emanation increases with the hyperemia of sexual organs in estrous cows, which is enhanced by close proximity of a bull (Hradecký et al., 1983).

The estrus-related odors have immediate (signaling) effects on animal behavior. The bull is attracted to estrous cows and shows his interest by sniffing, nuzzling, urine tasting, flehmen, and mounting (Hradecký et al., 1983). The paramount significance of odors in this behavior can be derived from the observations that even a sexually experienced bull can be confused and mount restrained nonestrous cows or dummies smeared with estrous vaginal secretions and urine (Hart et al., 1946; Donovan, 1967; Paleologou, 1977a,b). The chemical composition of the attractive odors apparently rapidly changes during estrus since the bull can precisely discriminate the olfactory information and gradually increase his attention towards estrous cows but reserves mounting for the standing heat (receptivity) only. The nonestrous penmates also respond to the estrous odors by mounting the estrous cows, and they too can be easily confused by the estrous odors transferred to nonestrous animals (Nishimura et al., 1983, 1984). The behavioral responses of penmates are apparently less discriminatory than those of the bull. This may explain the lower efficiency of estrus detection based on the observation of cow and heifer behavior in comparison with the heat checking using a sexually experienced bull.

Reproductive pheromones also have delayed (priming) effects on reproductive functions and metabolism. However, in large farm animals these effects have not been fully recognized since they are hidden in the generally defined complex of social and environmental influences on animal reproduction. Relevant experimental data are scarce because of difficulties with experimental design, such as definition of pheromone sources, exposure (temperature, ventilation, time and space relationships), long reproductive periods, and high costs involved. Nevertheless, reports do indicate involvement of the odorous cues in the regulation of reproductive functions in large farm animals. The sows exposed to synthetic boar pheromone androstenone improved their reproductive performance (Hillyer, 1976). Ram pheromones induced and synchronized estrus in sheep (Schinckel, 1954; Knight and Lynch, 1980; Martin et al., 1986) and goats (Shelton, 1980; Chemineau, 1983, 1987) early in the breeding season and increased their ovulation rates.

The presence of bulls in beef cow herds accelerated resumption of estrous cycles in postpartum cows (Zalesky et al., 1984; Kinder and Zalesky, 1985; Alberio et al., 1987). Unknown compounds from estrous cow urine and vaginal secretions improved synchronization of the estrus induced with prostaglandins in heifers (Izard, 1983; Izard and Vandenbergh, 1982; Vandenbergh and Izard,

1983). Estrous cows induced in nonestrous penmate cows urinary pH changes that were comparable with those in estrous cows and that might be related to pheromone production and release (Hradecký, unpublished data). Postpartum cows synchronized with progestagens apparently induced nearly synchronous estrus in nontreated control penmates, which may be another example of pheromonal influences in cows (Weston and Ulberg, 1976). Estrous cows and heifers also induced behavioral signs of estrus in herdmates without corresponding changes in reproductive organs and made estrus detection more difficult (Kedrov, 1952; Głód, 1961; Lojda, 1966, 1968).

Previous studies have linked the bull's flehmen response (Hradecký et al., 1983) and the emanation of attractive odors in cows (Akhlebininskii and Ishutov, 1974; Kiddy et al., 1978, 1984; Kiddy and Mitchell, 1981; Ladewig and Hart, 1981; Hawk et al., 1984) to the occurrence of estrus. The coincident increase in the olfactory attractivity of both estrous and nonestrous cows for the bull reported in this paper strongly suggests an involvement of olfactory cues and chemical communication (Hradecký et al., 1983). The observed attractivity of nonestrous penmates was clearly associated with the occurrence of estrus in another penmate cow and was reflected by the bull's increased attention and number of flehmen reactions.

The apparent ability of estrous cows to increase the attractivity of penmates is likely based on the same odorous cues that mediate chemical communication between cows and bulls. The unambiguous olfactory response of the bull to the estrous cows and much weaker but distinct and simultaneous olfactory response to both the penmate steer and the penmate nonestrous cows indicate the direction of supposed inductive effects from the estrous cows toward the penmates. The estrous cows produce and emit primary estrus-related attractive odors that may induce simultaneous emanation of secondary attractive odors in penmates. These secondary odors might be slightly different from the primary estrous odors and convey information different from that of the primary odors because the bull's attention was increased only moderately in comparison with his responses to primary odors, and the bull was not stimulated to mount.

The suggested odorous communication and induction effects among cows may correspond to analogous mutual influences in female groups of other species such as rats (McClintock, 1983, 1984), carnivores (Hradecký, 1985), red deer (Iason and Guinness, 1985), monkeys (Ruiz de Elvira et al., 1982, 1983), and humans (McClintock, 1971; Preti et al., 1986), although none of these reports has brought unambiguous direct evidence about the nature of the signals and sensory channels involved.

The steer was probably very susceptible to the induction effects of estrous penmates and easily responded by the emission of attractive odors. This emission seemed to last at least as long as the emission of primary odors in the estrous cows, as judged by the bull's behavior. The easy induction of attractive

odor emission and limited olfactory discrimination ability in steers may be involved in the etiology of buller steer syndrome in feedlots where about 2–3% of steers are persistently ridden by their penmates (Pierson et al., 1976; Brower and Kiracofe, 1978; Ulbrich, 1981). The buller steers may emit attractive odors and elicit mounting by the penmates. In extreme cases the buller steers might also induce odor production in other susceptible penmate steers and trigger mass mounting with likely adverse economic impact. These features also make the steer a valuable experimental model for future studies of pheromonal influences among cattle.

Chemical communication and pheromonal effects are as yet on the periphery of current knowledge in mammalian reproductive biology. Contemporary research trends in animal reproduction do not emphasize the pheromonal effects, but their existence does not contradict current knowledge. The exploration of chemical communication may offer alternative explanations for reproductive phenomena not yet adequately understood.

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QUANTITATIVE AND QUALITATIVE EFFECTS OF LARVAL DIET ON MALE SCENT SECRETIONS OF *Estigmene acrea*, *Phragmatobia fuliginosa*, and *Pyrrharctia isabella* (Lepidoptera: Arctiidae)

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Abstract—In feeding experiments with insects reared in the laboratory, the presence of the dihydropyrrrolizines hydroxydanaidal and danaidal in the male scent organs (coremata) of the arctiids, *Estigmene acrea* (Drury), *Phragmatobia fuliginosa* (L.), and *Pyrrharctia isabella* (J.E. Smith), was shown to depend on the presence of a source of pyrrolizidine alkaloids (PAs) in the larval diet. *Phragmatobia* males given an artificial diet supplemented with the powdered roots of the PA-containing plant *Symphytum officinale* L. (comfrey) produced more hydroxydanaidal than danaidal, whereas males given an artificial diet supplemented with dried whole plants of another PA-containing species, *Senecio vulgaris* L., produced more danaidal than hydroxydanaidal. *Pyrrharctia* males produced hydroxydanaidal with little if any danaidal, whether the source of PAs was comfrey or *S. vulgaris*. A behavioral bioassay showed that the coremata of PA-denied *Pyrrharctia* male progeny of PA-denied parents were pheromonally inactive, whereas those of PA-denied male progeny of PA-supplied parents (male and/or female) were often active. This indicates that a small amount of pheromone is made from PAs transferred from the female to her eggs and that males effect copulatory transfers of PAs that are, in turn, passed to the eggs by the mated female. Field observations of *Phragmatobia* and *Pyrrharctia* larvae feeding on sources of PAs were reported. The PA monocrotaline was shown to be a feeding stimulant for *Pyrrharctia* larvae.

Key Words—Diet, scent, coremata, *Estigmene acrea*, *Phragmatobia fuliginosa*, *Pyrrharctia isabella*, Lepidoptera, Arctiidae, hydroxydanaidal, danaidal, pheromone.

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INTRODUCTION

The plant secondary compounds known as pyrrolizidine alkaloids (PAs) are extremely widespread, currently known to occur in ca. 300 plant species in at least 11 families (Bull et al., 1968; Smith and Culvenor, 1981). Insects in four orders engage in "pharmacophagous" feeding on plants containing PAs (Boppré, 1986, and references cited), i.e., they ingest PAs for purposes other than primary metabolism or host-plant recognition (Boppré, 1984). This specialized feeding habit is widespread in two taxonomically distinct groups of Lepidoptera: the danaine and ithomiine butterflies (Nymphalidae) and the arctiid moths. Both groups include species in which adults obtain PAs by feeding through the proboscis on exudates of PA-containing plants (Pliske, 1975; Boppré, 1986, and references cited; Dussourd, 1986). The alkaloids are subsequently used as precursors for male scent secretions (Edgar et al., 1973; Schneider et al., 1975; Krasnoff and Dussourd, 1989, and references cited). There are also species in both groups in which PAs, subsequently used as pheromone precursors, are obtained from the larval host plant (Conner et al., 1981; Edgar, 1982; Boppré and Schneider, 1985, and references cited).

Previously we identified two dihydropyrrolizine pheromones, danaidal (Figure 1A) and hydroxydanaidal (Figure 1D), from the male scent organs (coremata) of two arctiids, *Phragmatobia fuliginosa* (L.) (hereinafter referred to as *Phragmatobia*) and *Pyrrharctia isabella* (J.E. Smith) (hereinafter referred to as *Pyrrharctia*) (Krasnoff et al., 1987), and showed that male pheromone titer varies widely in these species with most males having either ca. 1 μg of pheromone or less than 10 ng. Here we present evidence that the corematatal dihydropyrrolizines of these species as well as of another, *Estigmene acrea* (Drury) (hereinafter referred to as *Estigmene*), are also derived from PAs ingested by the larvae. We show that the pattern of variation in dihydropyrrolizine titer observed in *Phragmatobia* and *Pyrrharctia* is determined by differential feeding of larvae on PA-containing plants. For *Phragmatobia*, we show that the ratio of danaidal to hydroxydanaidal in the coremata varies with the species of PA-containing plant ingested by a larva. For *Pyrrharctia*, we present evidence that the pheromone precursors can be obtained from the parents in the absence of any larval access to PAs. We also report the association of *Phragmatobia* and *Pyrrharctia* larvae with sources of PAs in the field and present experimental evidence that PAs are feeding stimulants for *Pyrrharctia* larvae.

METHODS AND MATERIALS

Insects. Laboratory cultures were started with eggs obtained from females collected in the field at light traps in Ontario, Schuyler, Seneca, and Tompkins counties, New York State, during the summers of 1982, 1983, 1984, and 1985.

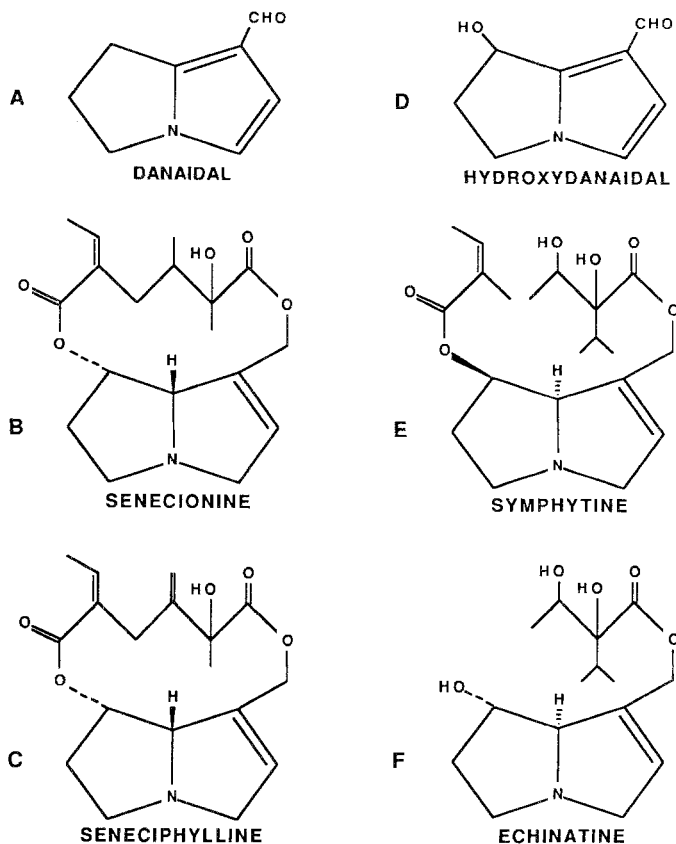


FIG. 1. Dihydropyrrolizidines (A,D); macrocyclic pyrrolizidine alkaloids (B,C); noncyclic diester and monoester pyrrolizidine alkaloids (E,F).

Larvae were reared in the laboratory on either natural plant material (*Arctium minus* (Hill) Bernh., *Taraxacum officinale* Weber, *Plantago lanceolata* L., *P. major* L., *P. rugellii* Dcne., *Polygonum cuspidatum* Sieb. & Zucc.), cultivated *Vicia faba* L. (fava bean), or an artificial pinto bean diet (Shorey and Hale, 1965). Larvae were held in a growth chamber at $25 \pm 2^\circ\text{C}$ under a 16:8 light-dark photoperiod. Records were kept of the diets of the parents of each brood in the second and subsequent generations removed from the field.

In addition, mature larvae were collected in the field during the fall and winter of 1983 and 1984 in Ontario, Steuben, and Tompkins counties, New York State. These larvae were maintained through winter diapause without food, according to the methods of Goettel and Philogène (1978). The following spring, the larvae pupated and adult moths were subsequently obtained.

Feeding Experiments. To investigate the effect of larval diet on male pheromone gland content, broods of larvae were divided into two groups. One (hereinafter referred to as PA-supplied) was offered a source of PA-laden food and the other (hereinafter referred to as PA-denied) was not. Larvae were supplied with PAs in one of several ways: (1) In addition to their primary food plant(s) or the artificial diet, larvae were given access to fresh material from a plant species either known to contain PAs (*Eupatorium maculatum* L., *Senecio vulgaris* L., *Symphytum officinale* L.) or presumed to contain PAs due to close taxonomic affiliation with a known PA-containing plant (*E. perfoliatum* L.) (Smith and Culvenor, 1981, and references cited). Control larvae were maintained on their original diet. (2) The dried roots of *Symphytum officinale* (comfrey), reported to contain PAs at ca. 0.1% by weight (Furuya and Araki, 1968; Furuya and Hikichi, 1971), were extracted in ethanol (100 g roots per 150 ml solvent) for 48 hr. The extract then was filtered and reduced in volume in a rotary evaporator and mixed thoroughly with a measured volume of pinto-bean diet so that a 1-gram-equivalent of roots was contained in 1.0 ml diet. Last-instar larvae feeding on the pinto bean diet were confined individually in 1-oz plastic cups with a 1.0-ml portion of treated diet. Controls were given a 1.0-ml portion of diet mixed with ethanol. Once the treated diet was consumed, larvae were returned to untreated diet and allowed to complete larval development. (3) A crude base fraction was obtained from the dried roots of comfrey using a modification of the methods of Furuya and Araki (1968) and Furuya and Hikichi (1971). These authors reported that the PA content of the crude base is 30–50%. Last-instar larvae feeding on fresh material from plants devoid of PAs were confined individually in 1-oz plastic cups with a leaf disk of the food plant treated with a known quantity of a 10% ethanol solution of the crude comfrey base. Controls were given a leaf disk treated with an appropriate amount of ethanol. After the disk had been completely consumed, larvae were returned to their previous food plant. (4) The dried roots of comfrey and oven-dried whole uprooted plants of *Senecio vulgaris* were ground to powders (20 mesh) that could be mixed into the pinto bean diet at a ratio of 15 g of powdered plant material to 500 ml of freshly prepared diet. These will hereinafter be referred to as comfrey powder and *Senecio* powder, respectively. At a known time after hatching, larvae feeding on the pinto bean diet were transferred to either the comfrey powder diet or the *Senecio* powder diet and allowed to feed until pupation. Controls were maintained on untreated pinto bean diet.

Corematal Chemistry. Extractions of coremata and subsequent mass spectral and gas chromatographic (GLC) analyses of corematal volatiles were carried out as described in Krasnoff et al. (1987).

Bioassay of Corematal Activity of PA-Denied Males. To determine whether males can use inherited PA precursors to make corematal pheromones, we adapted the experimental design used by Dussourd (1986) to demonstrate paren-

tal contribution of alkaloids to progeny in the arctiid *Utetheisa ornatrix* (L.). Comparisons were made of the stimulative capability of the coremata of PA-denied *Pyrharcitia* male progeny of (1) two PA-denied parents, (2) a PA-denied mother and a PA-supplied father, (3) a PA-supplied mother and a PA-denied father, and (4) two PA-supplied parents. A bioassay sensitive to picogram quantities of hydroxydanaidal (Krasnoff et al., 1987) was used. Male coremata were manually everted by gently squeezing the abdomen and were held ca. 2 mm from the antennae of a calling female for 5 sec. If the coremata elicited wing-fluttering and clicking in the female, a positive response was recorded (Figure 2). Each male was tested on one to four females, but each female was used only once with each male.

Larval Feeding on Pure Alkaloid. *Pyrharcitia* larvae (3 weeks old, third

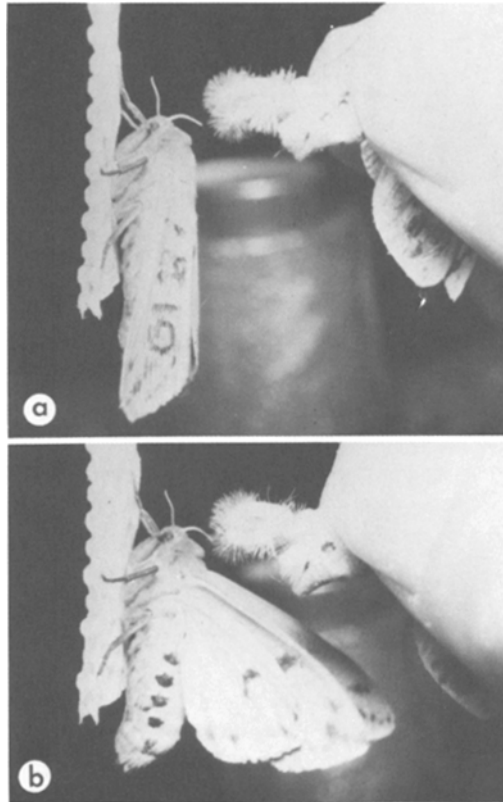


FIG. 2. Presentation of manually extruded *Pyrharcitia isabella* male coremata to a conspecific female: (a) immediately preceding onset of the wing-fluttering and clicking response; (b) during the response.

to fourth instar) were placed individually in waxed cartons (6 cm high \times 9 cm diameter). Two 24-mm Whatman No. 542 filter paper disks were positioned on insect pins ca. 2 mm above the bottom opposite each other along the edge of the carton. One disk was treated with 1 mg of the PA monocrotaline (Aldrich Chemical Co., Inc.) dissolved in 50 μ l of chloroform. The other disk was treated with 50 μ l of chloroform alone. After 24 hr each disk was assessed for evidence of feeding, and the disk that had been fed upon the most was determined. The test was replicated with 25 larvae.

RESULTS

Coremata Chemistry. Hydroxydanaidal was identified from *Estigmene*, *Phragmatobia*, and *Pyrrharctia*, and danaidal was identified from *Phragmatobia* by comparisons of GC retention times on polar and nonpolar capillary columns and by mass spectra of peaks in coremata extracts, with retention times and spectra from authentic samples of synthetic material.

Feeding Experiments. Due to mortality in rearing, sufficient numbers of insects were not obtained using the same protocols for all three species studied. Therefore data were presented for experiments in which sufficient sample sizes (ca. 10 insects in both treated and control groups) were obtained. In these experiments PA-supplied males of all three species produced two to four orders of magnitude more dihydropyrrolizines than their PA-denied counterparts (Table 1).

TABLE 1. EFFECT OF LARVAL DIET ON TOTAL DIHYDROPYRROLIZINE (HYDROXYDANAIDAL + DANAIDAL) CONTENT (ng \pm STANDARD ERROR) EXTRACTED FROM COREMATA

Species	PA-supplied	PA-denied	<i>P</i> ^a
<i>Estigmene acrea</i> ^b	18352 \pm 3770 (<i>N</i> = 9)	1 \pm 0.2 (<i>N</i> = 10)	<0.0001
<i>Phragmatobia fuliginosa</i> ^c	732 \pm 65 (<i>N</i> = 11)	5 \pm 1.2 (<i>N</i> = 10)	<0.001
<i>Pyrrharctia isabella</i> ^d	2717 \pm 377 (<i>N</i> = 10)	1 \pm 0.2 (<i>N</i> = 11)	<0.0001

^a *t* test performed on totals transformed to log₁₀.

^b PA-supplied were transferred from untreated pinto-bean diet to comfrey powder diet at three weeks after hatching (ca. eight days before spinning the pupal cocoon). Controls were maintained on untreated pinto-bean diet.

^c PA-supplied were given a 1-g equivalent of crude ethanolic comfrey root extract in 1 ml of fresh pinto-bean diet during the last instar. PA-denied were given 1 ml of diet mixed with ethanol during the last instar.

^d PA-supplied were given 1 mg of crude base from comfrey in 10 μ l ethanol on a 1.5-cm disk of a leaf of *Polygonum cuspidatum*. PA-denied were given a leaf disk treated with 10 μ l of ethanol. Larvae were 26 days old when the leaf disks were consumed.

Phragmatobia males reared on nonalkaloid plants but given a choice of PA plants in the last larval stage produced detectable amounts of dihydropyrrolizines. Those given *Eupatorium maculatum* had 63 ± 10 ng ($N = 6$, range: 34–88 ng), those given *Eupatorium perfoliatum* ($N = 2$) had 241 and 251 ng, and those given *Senecio vulgaris* had 292 ± 138 ng ($N = 3$, range: 62–541 ng). These *Phragmatobia* males produced pheromone profiles specific to the plants fed upon. Those given either of the *Eupatorium* species produced hydroxydanaidal, whereas those given *Senecio* produced more danaidal than hydroxydanaidal (Figure 3). *Phragmatobia* males reared on the *Senecio* powder diet produced significantly more danaidal than hydroxydanaidal ($P < 0.0001$, two-sided paired t -test), whereas males reared on the comfrey powder diet produced more hydroxydanaidal than danaidal ($P < 0.0001$, two-sided paired t -test) (Figure 4A).

In contrast, *Pyrrharctia* males did not show this effect. Both *Senecio*-powder-supplied males and comfrey-powder-supplied males produced more hydroxydanaidal than danaidal ($P < 0.001$, two-sided paired t -tests) (Figure 4B).

Inheritance of Pheromone Precursors. None of the PA-denied *Pyrrharctia* male progeny of PA-denied parents had pheromonally active coremata, whereas many of the PA-denied progeny of either one or two PA-supplied parents had active coremata (Table 2).

Larval Feeding on Pure Alkaloid. *Pyrrharctia* larvae showed a significant preference for the monocrotaline-treated filter paper over the filter paper treated with chloroform alone. Of the 25 *Pyrrharctia* larvae tested, 15 fed on the alkaloid-treated disk only, two fed on both, and eight fed on neither. Both of the larvae that fed on both disks fed more extensively on the monocrotaline-treated disk. The proportion feeding on monocrotaline (17/25) was significantly higher than the proportion that fed on the control (2/25) based on lack of overlap of the 99% confidence intervals of the proportions (Snedecor and Cochran, 1967, p. 210).

Association of Phragmatobia and Pyrrharctia Larvae with PAs in the Field. During October and November 1983, 1984, 1985, and 1986, last-instar *Phragmatobia* (ca. 5/year) and *Pyrrharctia* larvae (ca. 30/year) were collected on comfrey plants in a patch (ca. 500 m²) in Ontario County, New York State. A single *Pyrrharctia* larva was observed actually feeding on comfrey (Figure 5a). Also, during the winter and early spring, larvae were collected on comfrey under the matted-down litter of the previous year's foliage. Intensive searches of *Arctium minus* plants, which were interspersed among the comfrey plants in the study patch and produce a mat of foliage similar in mass and extent to comfrey, failed to yield any larvae. A last-instar *Phragmatobia* larva was observed feeding on the corpse of a *Halysidota tessellaris* J.E. Smith moth (Figure 5b). *H. tessellaris* is known to feed on PA-containing plants as an adult and may sequester PAs (Dussourd, 1986; Krasnoff and Dussourd, 1989).

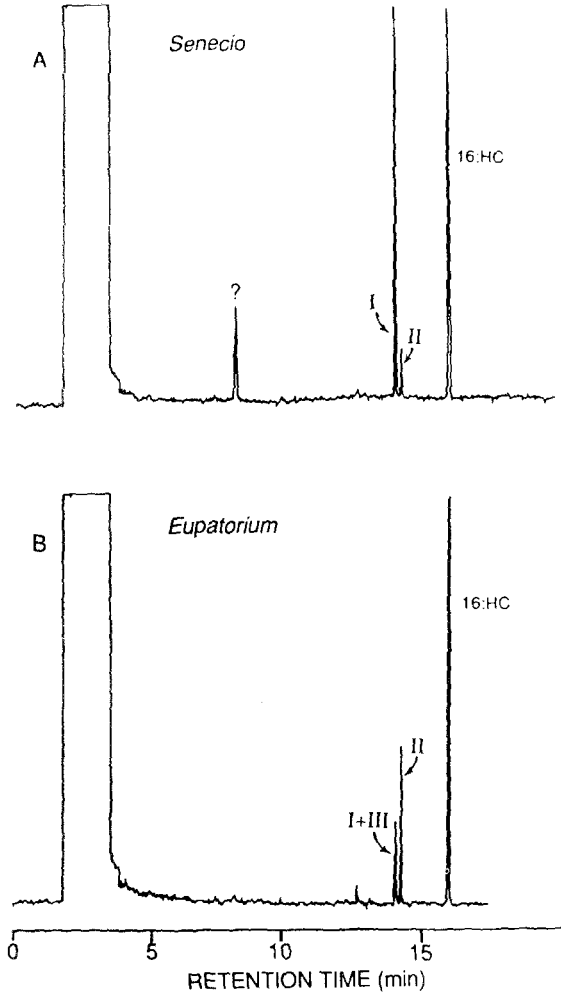


FIG. 3. Chromatograms of corematal pheromones of *Phragmatobia fuliginosa* males given access to the foliage of (A) *Senecio vulgaris* and (B) *Eupatorium maculatum*. Compound I is danaidal, compound II is hydroxydanaidal, compound III is a degradation product of II (see Krasnoff et al, 1987), and 16:HC is hexadecane, the internal standard. The peak marked by the question mark was unidentified. Estimates for male (A) were 252 ng for I and 21 ng for II. Estimates for male (B) were 31 ng I + III and 111 ng II.

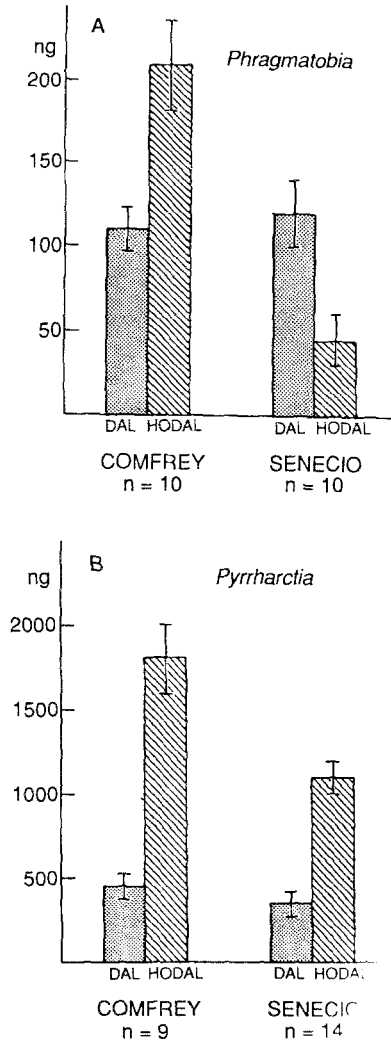


FIG. 4. Comparison of the effects of comfrey powder and *Senecio* powder diet additives on the relative amounts of hydroxydanidal (HODAL) and danaidal (DAL) in the coremata of (A) *Phragmatobia fuliginosa* males and (B) *Pyrrharctia isabella* males. Variation indicated is standard error of the mean.

TABLE 2. EFFECT OF PARENTAL FEEDING HISTORY ON COREMATAL ACTIVITY OF PA-DENIED *Pyrrharctia isabella* MALE OFFSPRING

Parental feeding history ^a		Males (N)	Response % ^b	100% ^c	0% ^d
Mother	Father				
PA-denied	PA-denied	15	0.0 (33) a	0.0	100.0
PA-denied	PA-supplied	27	81.8 (66) b	74.1	7.4
PA-supplied	PA-denied	2	100.0 (4) b	100.0	0.0
PA-supplied	PA-supplied	47	63.8 (116) b	34.0	19.2

^aPA-denied moths were reared on untreated pinto-bean diet. PA-supplied parents were reared on pinto-bean for 21 days after hatching and were then shifted to pinto-bean diet treated with comfrey powder.

^bThe manually everted coremata of each male were presented to one to four females, but each female was used only once with each male. Shown is the percentage of positive responses with the total number of trials pooled for all males of the designated type shown in parentheses. Percentages not followed by the same letter are significantly different at the 0.01 level according to Ryan's test of proportions (Ryan, 1960).

^cShown is the percentage of males that elicited a positive response from all females tested.

^dShown is the percentage of males that elicited no positive responses from all females tested.

Caterpillars collected from comfrey produced both male and female moths. The two *Phragmatobia* males taken as larvae from comfrey had 430 and 322 ng of total dihydropyrrrolizines. The *Pyrrharctia* males ($N = 5$) had 3430 ± 702 ng of pheromone ($N = 5$, range: 2009–5848 ng). A sample of *Pyrrharctia* larvae collected randomly (crossing roads) produced 14 males having 0–4132 ng (four males in the < 10 ng range, one each with 10–30, 30–100, 100–300, 300–1000 ng, four with 1000–3000 ng, and two with 3000–10,000 ng).

DISCUSSION

Besides adding *Phragmatobia*, *Pyrrharctia*, and *Estigmene* to the list of arctiids that derive dihydropyrrrolizine male scent secretions from PA precursors (Conner et al, 1981; Schneider et al, 1982; Boppré and Schneider, 1985), our findings are the first to demonstrate a qualitative effect of PA precursor plants on male pheromone chemistry in the Arctiidae. Culvenor and Edgar (1972) found qualitative variation in the relative amounts of hydroxydanaidal and danaidal in the coremata of *Utetheisa lotrix* (Cram.) in the field in Australia. These authors suggested that this variation might depend on the availability of different PA-containing plants (and consequently different PA precursors) in the different areas in which the two "types" of moths were collected. Our findings for *Phragmatobia* indirectly support this hypothesis. Males with more hydroxydan-



FIG. 5. (a) *Pyrrharctia isabella* larva feeding on a damaged leaf stem of a comfrey plant (October 1984). (b) A *Phragmatobia fuliginosa* larva feeding on the corpse of a *Halysidota tessellaris* moth. The larva continued to feed during and after transfer to the laboratory from the walk-in light trap in which it was discovered (photograph by John Truini).

aidal than dananidal, which is the pheromone ratio produced by feeding on *Eupatorium maculatum*, *E. perfoliatum*, or comfrey powder diet, are rare in the field (Krasnoff et al, 1987). One of the two wild *Phragmatobia* males taken from comfrey as a larva had more hydroxydananidal than dananidal, suggesting that it had indeed fed on the plant. The other had dananidal alone, suggesting that it had previously fed on some other PA plant, such as *Senecio vulgaris*, which results in more dananidal than hydroxydananidal—the pheromone ratio most commonly encountered in males from the field. It is apparent that *Phragmatobia* larvae “prefer” some PA-containing plants to others, at least in our study area. Further analyses of these and other related species may show that coremetal dihydropyrrolizine profiles can be used to determine both inter-specific and intraspecific patterns of variability in host-plant usage, much as

cardenolide profiles have been used to fingerprint monarch butterflies to particular milkweed species (Brower, 1984, and references cited).

Pyrrharctia males present an intriguing contrast to *Phragmatobia* males. The finding that *Pyrrharctia* males make the same blend of hydroxydanaidal and danaidal with both *Senecio vulgaris* and comfrey clearly demonstrates that the two species metabolize the same alkaloids in different ways. *Pyrrharctia* males almost always have more hydroxydanaidal than danaidal in nature but occasionally a field-collected male will have more danaidal than hydroxydanaidal (Krasnoff et al., 1987). This indicates that there is a PA-containing plant(s) in the field, rarely fed on by *Pyrrharctia* larvae, with alkaloids that produce more danaidal. Alternatively, there is a rare genetic variant of *Pyrrharctia* that metabolizes PAs in a different way.

In looking for a plausible structural relationship between the PA precursors and the two dihydropyrrolizines, we noticed that the primary alkaloid components of *Senecio vulgaris*, which are senecionine (Figure 1B), senecephylline (Figure 1C), integerrimine (Aplin et al., 1968), and retrorsine (Bull et al., 1968), are all macrocyclic diesters. In contrast, symphytine (Figure 1E) and echimidine, the principal alkaloids of comfrey (Furuya and Araki, 1968), and echinatine (Figure 1F) and lycopsamine, the principal alkaloids of *Eupatorium maculatum* (Tsuda and Marion, 1963; Resch, Goldstein, and Meinwald, unpublished data) and possibly of *Eupatorium perfoliatum*, are either monoesters or nonmacrocyclic diesters. *Phragmatobia* males made mostly danaidal from plants containing the macrocyclic PAs and made mostly hydroxydanaidal from plants containing the noncyclic PAs. It is clearly necessary to use pure alkaloids to investigate further the effect of precursor structure on pheromone composition.

Male pheromone titer in *Phragmatobia* and *Pyrrharctia* from the field is bimodally distributed, with many males having less than 10 ng of pheromone, but many also having 1 μ g or more (Krasnoff et al., 1987). The dihydropyrrolizine titers of the PA-supplied *Phragmatobia* and *Pyrrharctia* males in this study are on a par with those of field-collected males near the higher mode for their species, whereas the titers of PA-denied males resemble those of the field-collected males near the lower mode for their species (Krasnoff et al., 1987). For *Pyrrharctia*, males originally having microgram quantities of pheromone still have microgram quantities after exposure of the coremata (Krasnoff, 1987). Thus, for this species, the distribution of pheromone titer seen in nature is not due to differential use of the coremata but rather to differential feeding on PA-containing plants. We believe this conclusion can also be applied to *Phragmatobia*, which, in the manner of *Pyrrharctia*, everts the coremata in short intermittent bursts while searching for and courting a female (Nielsen, 1982; Krasnoff, 1987). Further support for this conclusion comes from the finding that a sample of males collected randomly as caterpillars (crossing roads) included individuals with 10 ng of pheromone or less as well as individuals

with 1 μg or more, whereas all those collected from a PA-containing plant (comfrey) were in the higher end of the distribution. Since these males were either unmated or had mated once in the laboratory before they were analyzed and had a maximum corematal eversion time of ca. 30 sec, their pheromone titer could not have been affected appreciably by corematal exposure.

In *Estigmene* as well as in the Asian arctiids *Cretonotos gangis* L. and *C. transiens* (Walker), the coremata are everted in a static courtship display that may last as long as 1.5 hr (Willis and Birch, 1982; Wunderer et al., 1986). In *Cretonotos hydroxydanaidal* attracts both males and females to a mating aggregation (Wunderer et al., 1986). A similar aggregation forms in *Estigmene*, but whether its formation is pheromonally mediated by hydroxydanaidal has yet to be determined (Willis and Birch, 1982). Preliminary analyses of field-collected males indicate that a pattern of variation in pheromone titer similar to that found in *Phragmatobia* and *Pyrharctia* also occurs in *Estigmene* (range 0–150 μg hydroxydanaidal; Krasnoff, unpublished). A broad variation in male pheromone titer also was reported in arctiids of the genus *Cretonotos* (Boppré and Schneider, 1985). In view of the protracted corematal display in these species, it is possible that loss of corematal compounds by exposure during mating could account for more of the variation observed in nature than with *Phragmatobia* and *Pyrharctia*. However, in *Cretonotos* spp. differential feeding also dramatically affects the size of male coremata, and the observation of both small and large coremata in natural populations (Boppré and Schneider, 1985) indicates differential feeding on PAs in the field in those species as well. Because no such morphogenetic effect was observed with *Estigmene* (or with *Phragmatobia* or *Pyrharctia*), proof that quantitative variation in the dihydropyrrolizine titer of wild *Estigmene* males depends on differential intake of PA precursors and not on differential loss from exposure of the coremata will have to await analysis of adults reared from field-collected larvae.

Females of several arctiid species are capable of passing PAs into their eggs (Benn et al., 1979; Dussourd, 1986; Boppré and Schneider, 1985; Dussourd et al., 1988). *Ciseps fulvicollis* Hübner and *Utetheisa ornatrix* males transfer PAs to females during copulation, and the alkaloids are then passed on to the eggs by the females (Dussourd, 1986). By showing that the feeding history of the parents affects the corematal chemistry of the male progeny in *Pyrharctia*, our bioassays provide indirect evidence for both inheritance of PAs and nuptial transfer in this species. Furthermore, they show that the trace amounts of dihydropyrrolizines detected in the PA-denied male progeny of field-collected females could have been made from PAs inherited from either parent or both. The relevance of these inherited pheromones to male mating success is discussed elsewhere (Krasnoff, 1987).

It is important to note that the species under consideration in this study, as well as the related *Cretonotos* spp. (Boppré and Schneider, 1985), are essen-

tially polyphagous (Forbes, 1960; Tietz, 1952, 1972; Shapiro, 1968), but associate nonrandomly in some as yet unknown way with PA plants in nature. Our experiments with *Pyrrharctia* and observations of all three species indicate that larvae will ingest pure PAs, as was reported for *Cretonotos* larvae (Boppré, unpublished, cited in Boppré and Schneider, 1985). Boppré and Schneider (1985) state that *Cretonotos* larvae can "detect" PAs, but they do not mention a mechanism of oriented search. The *Pyrrharctia* larvae in our experiment can also be said to have detected PAs in that they chose to feed on PA-impregnated filter paper over blank filter paper. However, it remains unclear whether there is a mechanism of orientation such as the upwind walking to PAs reported for the grasshopper *Zonocerus elegans* (Thunb.) (Boppré et al., 1984) or the upwind flight to PA-derived volatiles seen in some ithomiine butterflies (Pliske et al., 1976) and arctiid moths (Krasnoff and Dussourd, 1989). In preliminary experiments *Estigmene* larvae failed to exhibit upwind-oriented movement toward a PA source in a tube olfactometer. It may be that larvae of these generalist arctiid species are preferentially arrested by PA plants during repeated sampling of many different types of plants (Rothschild et al., 1979) but, lacking a mechanism of direct search, many larvae simply never find a plant source of PAs. This view is consistent with our previously reported pheromone data, which show that fewer than half of the *Phragmatobia* and *Pyrrharctia* males in nature ever feed on a PA-containing plant (Krasnoff et al., 1987).

Phragmatobia and *Pyrrharctia*, which thrive in nature without ingesting PAs and mate successfully with or without the intercession of a PA-derived male pheromone (Krasnoff, 1987), stand in distinct contrast to a species such as *Utetheisa ornatrix*, which oviposits and feeds exclusively on PA plants and has a PA-derived male pheromone that is a critical element of a successful courtship (Conner et al., 1981). There is an interesting parallel to this contrast in the danaiine butterflies. Pliske (1975) found that male pheromone release behavior (hairpenciling) occurs often in monarch courtship but is not necessary for mating to occur as it is in other danaiines such as the queen butterfly (Pliske and Eisner, 1969). Pliske also noted that the PA-feeding behavior seen in other danaiine butterflies (see Introduction above) is only weakly expressed in the monarch and that little if any PA-derived pheromone is present in the hairpencils. In this light Pliske considered the role of the male pheromone in monarch courtship to be reduced evolutionarily as a concomitant of a reduced dependence on PAs. We suggest that a similar evolutionary reduction has occurred in the Arctiidae. Species such as *Phragmatobia* and *Pyrrharctia* may exhibit an intermediate condition in a transformation from an ancestral PA-host-plant specialization (as seen in *Utetheisa*) to the polyphagous feeding habit typical of the arctiid lineage (Arctiinae: Arctiini) to which these species belong (Ferguson, 1985). There are several genera in this group (e.g., *Apantesis*, *Arctia*) that can be said to have lost the male pheromone system entirely by virtue of the fact

that the coremata have been lost (Ferguson, 1985). We suggest that the seemingly weak dependence on PAs manifested by *Phragmatobia* and *Pyrreharctia* is the vestige of an ancestral character that may be more fully developed in other, as yet unknown species in the lineage. It is hoped that further studies of host ranges, mating systems, and PA-derived courtship pheromones in other arctiids, combined with phylogenetic studies of the major lineages in the group, will eventually permit strong inferences to be made about the direction of evolutionary change in host range in this group, specifically regarding the history of the relationship with plants containing PAs.

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ATTRACTION OF ADULT SWEET POTATO WEEVILS,
Cylas formicarius elegantulus (SUMMERS), (COLEOPTERA:
CURCULIONIDAE), TO SWEET POTATO LEAF AND
ROOT VOLATILES

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Abstract—A dual-choice olfactometer was developed to study the responses of sweet potato weevils, *Cylas formicarius elegantulus* (Summers), to volatiles from the sweet potato, *Ipomoea batatas* (L.) Lam. Both males and females were attracted by volatiles from sweet potato leaves and a methylene chloride leaf extract. Females, but not males, responded to volatiles from storage roots and a methylene chloride root extract. Leaves and storage roots from four sweet potato cultivars (Centennial, Jewel, Resisto, and Regal) were attractive to female weevils; however, the attractant response varied with cultivar. GC profiles from leaf and root extracts, and GC-MS analysis of leaf extract, for Jewel cultivar enabled the volatile peaks to be identified as sesquiterpenes.

Key Words—Sweet potato weevil, *Cylas formicarius elegantulus*, Coleoptera, Curculionidae, *Ipomoea batatas*, sweet potato volatiles, sesquiterpenes.

INTRODUCTION

The sweet potato weevil, *Cylas* spp., is a serious pest of sweet potato, *Ipomoea batatas* (L.) Lam., worldwide, both in the field and during postharvest storage

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(Edmond, 1971; Sutherland, 1986). The oligophagous weevil consumes all parts of its host plant, while eggs are laid singly, mainly in the basal stem and storage roots (Cockerham et al., 1954). Some cultivars of sweet potato are moderately resistant to the weevil (Rolston et al., 1979; Mullen et al., 1985). A chemical basis for such resistance is possible, which may be effective at any stage of the weevil's host-location, host-selection, or host-utilization behavior.

Plant volatiles that act as insect attractants are known to occur widely (Finch, 1980; Visser, 1986). In a recent review, Metcalf (1987) listed 300 insect species in five orders that have been shown to respond selectively to 64 identified plant odorants. Little is known about the volatiles of sweet potato, although volatiles have been identified from baked storage roots (Purcell et al., 1980; Tui et al., 1985).

In this study, using two relatively susceptible cultivars, Centennial and Jewel, and two moderately resistant cultivars, Resisto and Regal (Mullen et al., 1985), the objectives were: (1) to determine whether male and female *Cylas formicarius elegantulus* (Summers) respond to host-plant volatiles from foliage or storage roots; (2) to see if the response is similar with cultivars having different levels of resistance to the weevil in the field, and (3) to identify the volatile components of sweet potato odor.

METHODS AND MATERIALS

Insect Rearing. Sweet potato weevils (*Cylas formicarius elegantulus*) were reared in the laboratory on sweet potato storage roots in cages held at 28°C, 65% relative humidity and 16:8 light-dark. Adult weevils were transferred to fresh potatoes every seven days, with the old potatoes being incubated until the new generation emerged. Emerging weevils were collected weekly and held in cages until required for bioassays. Weevils in the age range 18–34 days with no previous exposure to sweet potato foliage were used.

Leaf Material. All material selected was young, healthy fully extended sweet potato leaves of similar size, picked from the third of the vine nearest the growing tip. Greenhouse-grown leaves (Jewel cultivar), used in the initial bioassays, were harvested at 9:00 AM and placed immediately in test tubes of water so that they remained turgid. Field-grown leaf material was collected between 8:00 and 9:00 AM from plants grown at the University of Georgia Horticulture Farm. One leaf was taken from each plant sampled, and leaves were bioassayed on the day they were picked. All field-grown leaf bioassays were done in late August 1987. Leaves of greenhouse-grown *Plectranthus tuberosus* Poir Chev. et. Perrot (Senewiratne and Appadurai, 1966), an unrelated tuber-forming crop plant with a similar growth form to sweet potato, of equivalent surface area were used as a control. Attractant activity of *P. tuberosus*

was tested against test tubes of water (5 ml) as a control. Leaf areas were measured with a LI-COR (LI 3000) area meter.

Leaf surface extract for Jewel cultivar was obtained by agitating 1000 leaves individually for 10 sec each, in 3000 ml of methylene chloride (B & J distilled in glass grade). After filtering through a bed of sodium sulfate on Whatman No. 1 filter paper, the extract was reduced to 50 ml in a flash evaporator at 40°C, placed in a glass vial, sealed with a Teflon-lined cap and kept at 0°C until required. Known volumes of extract, thoroughly mixed and warmed to room temperature, were pipetted onto 1 × 1-cm squares of Whatman No. 1 filter paper. Solvent was evaporated. The extract was bioassayed against the equivalent volume of solvent alone. Fifty microliters (one leaf equivalent), 10 μ l and 1 μ l were tested for attractant activity.

Root Material. Small roots from four sweet potato cultivars were dug in the field at 8:00–9:00 AM on the day they were to be bioassayed. Roots were transported in brown-paper bags. Small potatoes (about 55 g) were bioassayed against a water control (5 ml in a test tube).

Root surface extract for Jewel cultivar was obtained by submerging recently cured storage roots in a beaker containing 3000 ml of methylene chloride (B & J distilled in glass grade) for 9 min in an ultrasonic bath (Son et al., 1987); this time was found to be the most efficient for extraction in preliminary studies (Son, unpublished data). The total weight of the roots extracted was measured on a top-loading balance. The methylene chloride extract was filtered through a bed of sodium sulfate and reduced to 45 ml over a steam bath using two 300-mm Kuderna-Danish concentrator columns. The extract was sealed in a glass vial with a Teflon-lined cap and kept at 0°C until required. The extract, warmed to room temperature, was pipetted onto Whatman No. 1 filter paper and the solvent evaporated off; 0.8 ml of extract was 1 potato equivalent, and a dose-response was obtained using 10, 1, and 0.1 potato equivalents.

Bioassays. The airflow to the olfactometer chambers (Figure 1) was controlled by a low-flow-rate air delivery system that has been described by Todd et al. (1977). The system employed a low positive-pressure flow regulated by a barostat tower and a series of capillary flowmeters. Prior to entering the flowmetering system, the air was humidified by bubbling it through warm water (30°C). A regulated airflow (20 ± 0.5 ml/min) was fed through tygon tubing (G) to each side of six olfactometers. Airflow in each tube was measured with a bubble flowmeter, and tubes with the nearest equivalent flow were attached to the two sides of the same olfactometer. The olfactometers consisted of a main chamber (A) (600 ml) (Figure 1) with a fitted lid, two gauze-covered holes, through which air exited the system, and a central baffle to reduce turbulence. Two glass connecting tubes (E) linked the main chamber with two half-pint glass Mason jars (200 ml, 6 cm diam × 9.5 cm), the treatment (B) and control (C) chambers. In the initial bioassays with greenhouse-grown leaves (Jewel

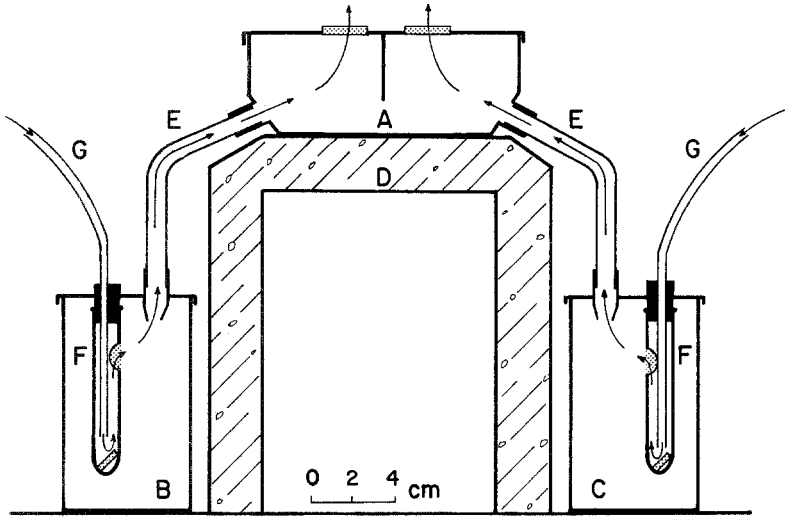


FIG. 1. Dual-choice olfactometer. Arrows indicate direction of airflow (20 ml/min each side). A, main chamber; B and C, treatment and control chambers; D, wooden stand; E, glass connecting tubes; F, test tubes with gauze-covered holes; and G, air input lines.

cultivar), the leaves were placed in side chambers (B or C), and weevils moving into these jars were counted. When field-grown leaves and roots of the four cultivars were bioassayed, extra identical glass jars were connected on each side of the olfactometer, with tygon tubing, in which the material was presented. When leaf and root extracts were bioassayed, glass test tubes (10 ml, 1.4 cm diam. \times 7 cm) (F) with 1.2-cm-diameter gauze-covered holes were placed in the side chambers (Figure 1). The extracts, dried on filter paper, were placed in these test tubes. Between experiments, the equipment was dismantled and washed with detergent, and the glassware washed with acetone.

Experimental Procedure. Twenty-five male or female sweet potato weevils of known age were placed in the main chambers (A, Figure 1) of each of six olfactometers, with the side arms sealed by tape at the main chamber ends, and placed in experimental conditions (28°C and 65–75% relative humidity) 2 hr prior to treatments being introduced. All experiments were done in darkness, to ensure that the weevils were behaviorally active (Cockerham et al., 1954; Proshold, 1983). The chambers were carefully leveled, as an incline can affect weevil distribution (Mullen et al., 1980). To initiate olfactometer tests, weevils were given access to the side chambers by simultaneously removing the seals from the glass connecting tubes. All experiments were started at 2:00 PM and lasted for 2 hr, after which the numbers of weevils in the treatment and control chambers (B and C, Figure 1) were counted. An additional experiment was

done to see if the presence of weevils of the same sex and age ($n = 10$ weevils) in the side chamber attracted test weevils compared with a blank control.

Statistical Analysis. The numbers in the treatment and control chambers for each treatment replicate were analyzed using a paired Student's t test. An attraction index was also calculated for each replicate $[(T - C)/N \times 100]$, where T is the number of weevils in the treatment chamber, C is the number in the control chamber, and $N = 25$, the number of weevils originally in the main chamber] (Tipping et al., 1987). Paired t tests were done to compare attraction indices between treatments.

Chemical Analysis. The methylene chloride extracts of leaves and roots, as used in the olfactometer bioassays, were analyzed by gas chromatography. Volatiles were also collected from leaf material by a "purge-and-trap" technique (Easley et al., 1981). Fifty leaves (Jewel and Regal cultivars) picked from field plots (8:00–9:00 AM) were placed in a 1000-ml Wheaton purging unit, in a 60°C water bath. Volatile collection commenced at 9:30 AM by passing nitrogen (50 ml/min) through the leaf material and out through an initial water trap, then into pentane (B & J distilled in glass grade) in two 300-ml impingers, placed in series and cooled in beakers of ice. Collection was continued for 3 hr, after which pentane was reduced in volume to 5 ml with a Kuderna-Danish concentrator. Extract was stored at 0°C.

The methylene chloride and "purge-and-trap" extracts were analyzed for volatile components using a Hewlett-Packard 5790 gas chromatograph with 0.32-mm-ID \times 30-m OV-225 fused silica capillary column, using splitless injection and flame ionization. Temperature was initially at 30°C for 2 min then increased at 4°C/min to 200°C. GC-MS analysis was conducted on a Hewlett-Packard S985 modified for capillary GC-MS (Arrendale et al., 1984). *trans*-Caryophyllene and α -humulene were obtained from Fluka Chemical Corporation.

RESULTS

Both male and female weevils were attracted by volatiles given off from sweet potato leaves (Jewel cultivar) (Table 1, A), and the level of response was similar. No response was obtained when the non-host-plant control *P. tuberosus* was tested against a water control (Table 1, B) for either males or females. Female weevils were attracted by volatiles from leaves of four sweet potato cultivars (Table 2). However, the level of the attractant response was higher for Jewel and Resisto leaves than for Regal leaves.

Females and males were both attracted to 1 and 0.2 leaf equivalent concentrations of Jewel leaf extract, but not to a 0.02 leaf equivalent concentration (Table 3). The level of attraction (attraction indices) was similar for males and females.

TABLE 1. (A) JEWEL SWEET POTATO LEAF AND *P. tuberosus* CONTROL, AND (B) *P. tuberosus* LEAF AND WATER CONTROL CHAMBERS OF OLFACTOMETER^a

	Surface area of leaves (cm ²)	No. weevils/chamber		Attraction index
		Sweet potato	<i>P. tuberosus</i>	
A. Sweet potato leaf and <i>P. tuberosus</i>				
Female				
Sweet potato	40.7 ± 2.8	10.7 ± 1.2	0.3 ± 0.3***	41.6a
<i>P. tuberosus</i>	51.0 ± 6.9			
Male				
Sweet potato	53.3 ± 2.2	14.8 ± 0.4	3.5 ± 1.0***	45.2a
<i>P. tuberosus</i>	51.3 ± 3.0			
		<i>P. tuberosus</i>	Water	
B. <i>P. tuberosis</i> and water				
Female				
<i>P. tuberosus</i>	48.1 ± 4.7	0.2 ± 0.2	2.3 ± 0.8	-8.4b
Male				
<i>P. tuberosus</i>	50.4 ± 1.7	1.2 ± 0.4	1.7 ± 0.7	-2.0b

^aWeevils were all aged between 21 and 28 days. $N = 6$, ± SE, 25 insects/replicate. Differences between numbers in two chambers of olfactometer, *** $P < 0.001$; treatments followed by different letters have significantly different attraction indices, $P < 0.05$, paired t tests.

TABLE 2. NUMBERS OF FEMALE SWEET POTATO WEEVILS, *Cylas formicarius elegantulus*, IN TREATMENT (SWEET POTATO LEAF) AND CONTROL (*P. tuberosus* LEAF) CHAMBERS OF OLFACTOMETER FOR FOUR SWEET POTATO CULTIVARS^a

Cultivar	Surface area of leaves(cm ²)		No. weevils/chamber		Attraction index
	Sweet potato	<i>P. tuberosus</i>	Sweet potato	<i>P. tuberosus</i>	
Centennial	84.6 ± 3.6	86.9 ± 6.8	9.7 ± 3.4	1.3 ± 0.7*	34.0ab
Jewel	77.9 ± 4.0	76.7 ± 1.8	13.2 ± 1.8	1.5 ± 0.8**	46.7a
Resisto	60.0 ± 4.0	58.1 ± 2.1	11.7 ± 2.1	1.5 ± 0.7*	40.7a
Regal	78.3 ± 3.7	74.9 ± 2.7	6.0 ± 0.9	1.0 ± 0.5*	19.3b

^aWeevils were 18–34 days old. $N = 6$, ± SE; 25 insects/replicate. Differences between numbers in two chambers of olfactometers, * $P < 0.05$ and ** $P < 0.01$; treatments followed by different letters have significantly different attraction indices, $P < 0.05$, paired t tests.

TABLE 3. NUMBERS OF FEMALE AND MALE SWEET POTATO WEEVILS, *Cylas formicarius elegantulus*, IN TREATMENT (JEWEL LEAF EXTRACT) AND CONTROL (SOLVENT ONLY) CHAMBERS OF OLFACTOMETER^a

Leaf equivalents	No. weevils/chamber		Attraction index
	Sweet potato	Control	
Female			
1	10.0 ± 1.5	3.3 ± 0.8**	26.8a
0.2	8.8 ± 1.3	1.0 ± 0.5**	31.2a
0.02	8.8 ± 2.0	5.8 ± 2.1	12.0b
Male			
1	12.8 ± 1.6	3.8 ± 1.1**	36.0a
0.2	13.2 ± 2.1	4.7 ± 1.2*	34.0a
0.02	2.3 ± 0.6	1.0 ± 0.4	5.2b

^aWeevils used were 21–34 days old. $N = 6$, \pm SE, 25 insects/replicate. Differences between numbers in two chambers of olfactometer, * $P < 0.10$, ** $P < 0.05$; treatments with different superscript letters have significantly different attraction indices, $P < 0.05$, paired t tests.

Female weevils were attracted by volatiles from storage roots of all four sweet potato cultivars (Table 4), with the attractant response to Jewel being significantly higher than to Resisto. Male weevils were not attracted by volatiles from storage roots of either Centennial or Jewel cultivar (Table 4).

Females were attracted by volatiles from three concentrations of Jewel root extract (Table 5), with the attractant response to 10 root equivalents being higher than that to 0.1 root equivalents. Males were not attracted to volatiles from Jewel root extract (Table 5).

No significant attraction was obtained to volatiles emitted from 10 weevils of the same sex in the treatment chamber (bioassayed against a blank control) for females (attraction index 4.8) or males (attraction index 2.8).

The methylene chloride leaf and root extracts from Jewel cultivar gave GC profiles with a number of peaks in the volatile range (Figure 2). The “purge-and-trap” volatile extracts from Jewel and Regal leaves showed the same volatile peaks; the Jewel leaf GC profile (Figure 2, top) had peaks that coincided with these in the methylene chloride leaf and root extracts; although the volatile components of the methylene chloride leaf extract may not have been trapped as efficiently due to its method of preparation. The “purge-and-trap” leaf volatile extract was subjected to GC-MS analysis, and all components eluting between peaks 1 and 7 (Figure 2) had molecular ions at m/e 204 and yielded spectra characteristic of sesquiterpenes. Peaks 1–5 (Figure 2) were tentatively

TABLE 4. NUMBERS OF FEMALE AND MALE SWEET POTATO WEEVILS, *Cylas formicarius elegantulus*, IN TREATMENT (SMALL SWEET POTATO ROOT) AND CONTROL (WATER) CHAMBERS OF OLFACTOMETER, FOR FOUR AND TWO SWEET POTATO CULTIVARS, RESPECTIVELY^a

Cultivar	Weight (g)	No. weevils/chamber		Attraction index
		Sweet potato	Control	
A. Female				
Centennial	51.8 ± 5.6	9.0 ± 1.7	1.5 ± 0.5**	30.0ab
Jewel	55.7 ± 5.1	13.0 ± 1.8	0.5 ± 0.2***	52.0a
Resisto	59.3 ± 2.1	7.5 ± 2.0	0.8 ± 0.4**	26.7b
Regal	57.4 ± 2.7	9.3 ± 2.3	1.0 ± 0.5*	33.3ab
B. Male				
Centennial	49.6 ± 2.2	5.1 ± 1.2	3.3 ± 0.9	7.2c
Jewel	49.1 ± 5.8	2.3 ± 0.7	0.6 ± 0.3	6.8c

^aWeevils used were 18–32 days old. (A) $N = 6$, (B) $N = 12 \pm SE$, 25 insects/replicate. Differences between numbers in two chambers of olfactometer, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; treatments followed by different letters have significantly different attraction indices, $P < 0.05$, paired t tests.

TABLE 5. NUMBERS OF FEMALE AND MALE SWEET POTATO WEEVILS, *Cylas formicarius elegantulus*, IN TREATMENT (JEWEL ROOT EXTRACT) AND CONTROL (SOLVENT ONLY) CHAMBERS OF OLFACTOMETER^a

Potato equivalents	No. weevils/chamber		Attraction index
	Sweet potato	Control	
A. Female			
10	10.3 ± 1.3	2.5 ± 0.4**	31.2a
1	9.5 ± 1.0	3.3 ± 0.7*	24.8ab
0.1	4.2 ± 0.4	1.5 ± 0.7*	10.8b
B. Male			
10	2.1 ± 0.4	1.4 ± 0.4	2.8c
1	3.9 ± 0.7	3.5 ± 1.0	1.6c

^aWeevils used were 18–32 days old. (A) $N = 6$, (B) $N = 12, \pm SE$, 25 insects/replicate. Difference between numbers in two chambers of olfactometer, * $P < 0.05$, ** $P < 0.01$; treatments followed by different letters have significantly different attraction indices, $P < 0.05$, paired t tests).

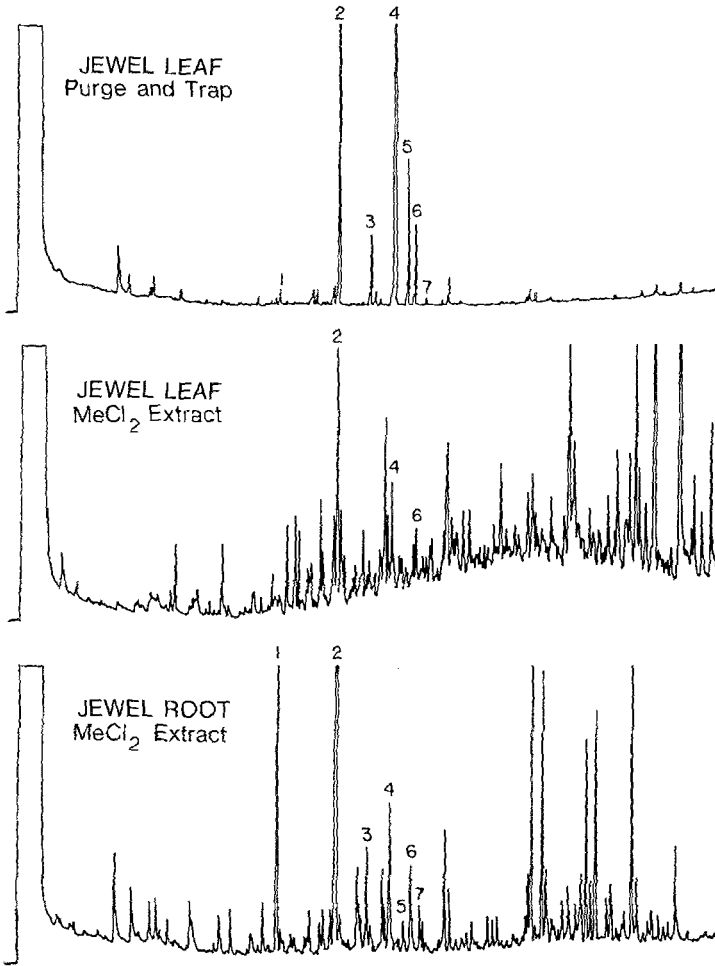


FIG. 2. GC profiles of "purge-and-trap" Jewel extract, methylene chloride Jewel leaf extract, and methylene chloride Jewel root extract.

identified from their MS spectra using reference data (Stenhagen et al., 1974) as: peak 1, copaene; peak 2, *trans*-caryophyllene; peak 3, α -humulene; peak 4, γ -cadinene; and peak 5, γ -elemene. The identity of peaks 2 and 3 were also confirmed by comparison with mass spectral and GC retention times of authentic samples.

DISCUSSION

The results showed that sweet potato weevils respond behaviorally to host-plant volatiles. Both sexes were responsive to leaf volatiles, which is not surprising when it is considered that host plants act as feeding and mating sites for the weevils (Proshold, 1983). Females, but not males, were responsive to root volatiles. Previous data have shown that male and female weevils feed to an equal extent on leaves (Nottingham et al., 1988), but females feed to a greater extent than males on root cores (Nottingham et al., 1989); in culture conditions females are predominantly observed on the storage roots, with males being more frequently observed elsewhere around the cages. Therefore, both sexes may utilize host-plant foliage to a similar extent, but females may utilize the storage roots more than males, explaining the lower attractant response of males to root volatiles. Volatiles from the roots might contribute to the oviposition stimulation response of female weevils in the presence of storage root periderm (Nottingham et al., 1987).

Male and female weevils did not appear to be attracted to volatiles from weevils of the same sex. This was also found to be the case by Coffelt et al. (1978). This suggests that male-male or female-female aggregation pheromones might not occur in this species, although further work will need to be done to confirm this. Male weevils, however, are attracted to a female sex pheromone (Coffelt et al., 1978; Heath et al., 1986). Interactions between host-plant odor and pheromone may occur in the field, influencing host-selection behavior, e.g., for plants on which females are "calling." Males also appear to fly more freely than females (Deen, 1940) are caught more frequently in light traps than females (Cockerham et al., 1954), and fly to pheromone traps, particularly in the hours just before dusk (Proshold et al., 1986). A similar anemotactic behavior to that which males use to locate pheromone traps (Proshold et al., 1986) might also be used to locate the source of host-plant volatiles in conjunction with plant visual cues.

Jewel cultivar volatiles were more attractive to female weevils than Regal cultivar for the leaves, and Resisto cultivar for the roots. The highest attraction index occurred for Jewel cultivar for leaf and root volatiles. Differences in the relative levels of resistance of sweet potato cultivars to the weevil may have a chemical basis. For example, differences in the levels of feeding and oviposition on root cores of the same four cultivars as used in this study were observed (Nottingham et al., 1987), which corresponded with differences in the root surface chemistry of the cultivars (Nottingham et al., 1989). Differences in host-plant volatiles between cultivars might also contribute to the overall level of cultivar resistance. Volatile profiles of host plants are also likely to change with foliage age and host-plant condition and may affect weevil host-selection behavior accordingly (Finch, 1980).

The analysis of sweet potato volatiles showed that a number of similar sesquiterpenes were associated with the leaves and roots, although relative levels differed. All the major volatile peaks appeared to be sesquiterpenes, which therefore are likely to be involved in the attractant response of the sweet potato weevil. Sesquiterpenes are known attractants of other Coleoptera (Metcalf, 1987); for example, γ -cadinene is one of the attractants of the elm bark beetle, *Scolytus multistriatus* (Marsham) (Col.; Scolytidae) (Millar et al., 1986), while β -caryophyllene, along with β -bisabolol, are host-plant attractants of the boll weevil, *Anthonomus grandis* Boh. (Col.; Curculionidae) (Dickens, 1984).

Further work will aim to bioassay the sesquiterpenes identified, individually and in combinations, in order to identify the specific host-plant attractants of the sweet potato weevil.

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Book Review

Special Issue on Pheromones (*Agriculture, Ecosystems and Environment*).
F.J. Ritter (ed.). 1988. U.S. \$59.00. Volume 21. 133 pages.

This special issue of *Agriculture, Ecosystems and Environment* is drawn together by guest editor F.J. Ritter from papers and posters presented in a symposium entitled "Pheromones and Other Behavior Modifying Chemicals" at the International Exhibition and Congress "Parasitis 1986" held late in 1988 in Switzerland. What makes this issue "special" beyond its bibliographic complexity? For JAEE, a strict focus upon agricultural chemical ecology is unusual. The content ranges from typical *Journal of Chemical Ecology* applied contributions of successfully implemented mass trapping and mating disruption programs to several disjointed collections of loosely related projects swept together under a general heading. Several countries whose research is less commonly seen in Europe and the United States are represented, such as Egypt and Peru, making some of the citation lists useful to those of us on different continents. What *is* special about this issue is the publisher's exorbitant asking price: \$59 (softcover)! Alternatively, the issue is received gratis by subscription to the *Journal of Agriculture, Ecosystems and Environment*.

None of the papers published in this issue represent either thorough reviews or conceptual advancements in applied chemical ecology. My advice to the reader is to buy the few stamps needed for mailing reprint requests for the one or two papers of interest.

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OLFACTORY RESPONSES OF *Orius insidiosus* (Hemiptera: Anthocoridae) TO VOLATILES OF CORN SILKS

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Abstract—A synomone present in hexane extracts of corn silks was found to attract *Orius insidiosus* (Say). The attraction was a diurnal, innate behavior, independent of sex. A portion of the prey-searching behavior of *O. insidiosus* apparently relies on olfactory perception when corn is the prey's host.

Key Words—*Orius insidiosus*, Hemiptera, Anthocoridae, attraction, synomone, plant volatiles, corn silks.

INTRODUCTION

Orius insidiosus (Say), the minute pirate bug, is a polyphagous predator that feeds on several corn-related insects including the eggs of *Ostrinia nubilalis* (Hübner), the European corn borer (Bartholomai, 1954; Jarvis and Guthrie, 1987), and *Heliothis zea* (Boddie), the corn earworm (Barber, 1936). *O. insidiosus* may have the potential to assist in the control of some lepidopterous pests of corn.

One method for augmenting natural enemies is to manipulate their abundance and field distribution through the use of attractants and feeding stimulants (Nordlund et al., 1981). The volatiles of host plants, for example, often help predaceous insects locate habitat and prey (Greany and Hagen, 1981).

Several studies have found a variety of volatile compounds in corn leaves, husks, tassels, and whorls (Thompson et al., 1974; Buttery et al., 1978, 1980). Flath et al. (1978) identified 63 volatile components in corn silk. Cantelo and Jacobson (1979) identified nine volatiles from corn silks that attract the corn

borer and corn earworm. The present study was designed to investigate the possibility that corn silks might attract adult *O. insidiosus*.

METHODS AND MATERIALS

Preparation of Corn Silk Extracts. A Waring blender was used to homogenize 30 g fresh weight of corn silks in 200 ml of water, ethanol, methylene chloride, or hexane. The corn silk extracts were then filtered through a Buchner funnel lined with filter paper. Each extract was dried with anhydrous magnesium sulfate and refiltered to remove the hydrated salts. A rotary vacuum evaporator was used to reduce the volume of each extract to a volume of 25 ml (i.e., 1.2 g silk equivalent/ml of solvent). In one experiment, a 20-g silk equivalent/ml hexane extract was used and was derived from 20 g of silk as described above.

Bioassay Procedure. The responses of *O. insidiosus* adults to the extracts were tested in an olfactometer using the Y-tube principle (McIndoo, 1926). A 90-cm black rubber hose leading from an air pump was connected to the leg of a Y-shaped glass tube. From each arm of the Y-shaped tube, a 30-cm length of clear plastic hose was connected to a 50-ml side arm flask. A second 30-cm length of clear plastic hose connected each flask to one of the arms of a second Y-shaped glass tube. To prevent the adult *O. insidiosus* from moving past the arms of the olfactometer, a piece of drapery sheer was used to cover the arm ends of the second Y-shaped tube. The adults were introduced at the base of the Y of the second tube and given a maximum of 10 min to respond. If an adult moved into one of the olfactometer arms up to the drapery sheer insert, a positive response to the substance in the connecting side-arm flask was recorded. If, after 10 min, the adult remained at the lower end of the Y-shaped tube, "neither" was recorded as the response. After 10 min, the hoses of each side-arm flask were reversed on the olfactometer, the same adults reintroduced, and the observations repeated.

All tests were conducted at 25°C in a room under natural light from 1330 to 1430 hr. The *O. insidiosus* used in all but one experiment were from a laboratory colony raised on *Pseudoplusia includens* (Walker) eggs with snapbeans as an ovipositional substrate. In the fourth experiment adults were collected from a corn field.

The Test Series. A series of olfactometer tests were run to determine whether or not a kairomone existed in corn silks that was attractive to *O. insidiosus*.

The first test run was to investigate if *O. insidiosus* was attracted to any odor given off by corn silks. Five grams of fresh silk were placed in one side-arm flask and one of the following was placed in the other flask: 0.5 g of corn

pollen, 1 corn leaf crushed in 5 ml of water, or 0.5 ml of vanilla extract. Vanilla extract was used to determine if *O. insidiosus* merely responded to odors in general. This experiment used five adults and was replicated twice.

In the second, third, and fifth experiments, 1 ml of a 1.2-g silk equivalent/ml of solvent extract was placed on filter paper and allowed to dry for about 2.5 min to remove excess solvent. The filter paper was then placed in the side-arm flask. Each replicate used six adults in these three experiments.

The second set of experiments determined which of a series of silk extracts was more attractive to *O. insidiosus* than a water control. One milliliter of water was placed on filter paper. While still moist, the paper was placed in the other side-arm flask. This experiment was replicated 10 times, and six adults were used in each replicate.

The third experiment was conducted to determine if an attraction to an extract was the result of the extract components or of residual solvent in the extract. One milliliter of pure solvent was put on filter paper and allowed to dry. The paper was then placed into a side-arm flask. This experiment was replicated four times for each of the solvents, excluding water. Six adults were used in each replicate.

The fourth experiment compared the attractiveness of the hexane extract to that of fresh corn silks. Twenty grams of fresh corn silk were placed in one side-arm flask and 1 ml of a 20-g silk equivalent/ml hexane extract was placed in the other. The experiment used 10 adults and was replicated eight times. This experiment was also repeated using *O. insidiosus* collected in the field.

The final experiment compared the response of male and female *O. insidiosus* to the hexane extract; water was used as the control. Tests were conducted at 1330 hr and 2130 hr to check for diurnal rhythms of activity. This experiment was replicated six times over a three-day period. Six adults were used in each replicate.

Statistical Procedure. The second, third, and fourth experiments were statistically analyzed using the paired sign test (Steel and Torrie, 1980). The final experiment was analyzed using a 2×2 contingency table (Sokal and Rohlf, 1969). Insects that failed to choose either arm of the olfactometer were omitted from the analysis.

Extract Analysis. The hexane extract of the corn silks was injected onto a coupled Hewlett Packard 5995 GC-MS (electron impact, 70 eV) interfaced with a HP 59970A GC-MS computer system with an NBS mass spectral library containing spectra of over 38,000 compounds. The column was a fused silica capillary column (10 m \times 0.2 mm) with a cross-linked methyl silicone phase (Hewlett Packard, Rolling Meadows, Illinois), and helium was used as the carrier gas. The temperature program was 40°C to 250°C at 10°C per minute with a 2-min hold; injector transfer line, and ion sources were set at 230°C, 200°C, and 220°C, respectively. Authentic phenylacetaldehyde was purchased from

Aldrich Chemical Co. (Milwaukee, Wisconsin) and injected under the same conditions on the GC-MS.

RESULTS AND DISCUSSION

In the first test, 100% of 10 *O. insidiosus* adults were attracted to corn silks; none were attracted to the crushed corn leaf, the corn pollen, or the vanilla extract.

The corn silk extracts made with hexane and methylene chloride attracted significantly more *O. insidiosus* adults than did pure water (Table 1). Water alone attracted significantly more *O. insidiosus* than did corn silk extracts in ethanol or in water (Table 1). These data suggest a strong response either to the lipophilic components in corn silks or to the solvents hexane and methylene chloride. It is also possible that the ethanol and water extracts may contain a repellent.

Table 2 shows that the hexane extract attracted significantly higher numbers of *O. insidiosus* than did pure hexane solvent. Attraction to the ethanol and methylene chloride extracts were not significantly different from attraction to their respective solvents (Table 2). In an earlier test, methylene chloride extract attracted a significant number of adults (Table 1); the absence of significance in this experiment could be due to small sample size because over 40% of the adult *O. insidiosus* failed to respond to the methylene chloride extract or to pure methylene chloride. The response to the hexane extract is of particular interest; 95.8% of the adults were attracted, strongly suggesting that *O. insidiosus* responds to volatiles in the hexane extract of the corn silks.

Table 3 shows that naive laboratory-reared and field-collected *O. insidiosus* adults did not differ significantly in their attraction to corn silk. This result

TABLE 1. RESPONSE IN THE LABORATORY OF ADULT *O. insidiosus* TO VARIOUS CORN SILK EXTRACTS VS. WATER

Corn silk extracted in	Number of <i>O. insidiosus</i> ^a		
	Attracted to extract	Attracted to neither	Attracted to water
Water	8	15	37**
Ethanol	3	20	37**
Methylene chloride	31*	12	17
Hexane	50**	10	0

^aPooled data for 10 replications of six adults each. *Significant at $P < 0.05$; **significant at $P < 0.005$. Paired sign test (Steel and Torrie, 1980).

TABLE 2. RESPONSE IN THE LABORATORY OF ADULT *O. insidiosus* TO VARIOUS CORN SILK EXTRACTS VS. EXTRACT SOLVENT

Corn silk extracted in	Number of <i>O. insidiosus</i> ^a		
	Attracted to extract	Attracted to neither	Attracted to solvent
Ethanol	3 ^b	15	6
Methylene chloride	9 ^b	10	5
Hexane	23 ^c	1	0

^aPooled data for four replications of six adults each.

^b0.5 > *P* > 0.1. Paired sign test (Steel and Torrie, 1980).

^cSignificant at *P* < 0.05. Paired sign test (Steel & Torrie, 1980).

suggests that the attraction could be an innate behavior. Furthermore, their attraction to the hexane extract did not differ significantly from their attraction to fresh corn silk. This finding indicates that a component of the hexane extract is as effective in attracting adult *O. insidiosus* as the synomonal substances in corn silk.

Significantly higher numbers of adult *O. insidiosus* were attracted to the hexane extract at 1330 hr than at 2130 hr (Table 4). This finding suggests a diurnal rhythm in the response. In fact, no significant attraction occurred at 2130 hr, and most insects failed to enter either arm of the olfactometer. Of those that did, approximately equal numbers were attracted to water and to the hexane extract. The attraction of male *O. insidiosus* to the hexane extract at 1330 hr was not significantly different than that of females, a finding that indicates that attraction is independent of sex.

The preliminary analysis of the hexane extract of the corn silks indicated

TABLE 3. RESPONSE IN THE LABORATORY OF ADULT *O. insidiosus* TO CORN SILK VS. CORN SILK HEXANE EXTRACT

Kind of adult	Number of <i>O. insidiosus</i> ^a		
	Attracted to silk	Attracted to neither	Attracted to extract
Naive	38	7	35 ^b
Field	38	8	34 ^b

^aPooled data for eight replications of 10 adults each.

^b0.9 > *P* > 0.05. Paired sign test (Steel and Torrie, 1980).

TABLE 4. EFFECT IN THE LABORATORY OF SEX AND TIME OF DAY ON ATTRACTION OF ADULT *O. insidiosus* TO HEXANE EXTRACT VS. WATER

Time (hr)	Number of <i>O. insidiosus</i> ^a					
	Attracted to hexane		Attracted to neither		Attracted to water	
	Male	Female	Male	Female	Male	Female
1330 ^b	14	17	0	0	4	1
2130 ^b	1	5	13	10	4	3

^aPooled data for six replications of six adults each.

^b $P \ll 0.005$. 2×2 contingency table, using the G statistic (Sokal and Rohlf, 1969).

compounds eluting after approximately 170°C were primarily waxes and long-chain hydrocarbons from the cuticular surface of the corn silks. Of the 16 compounds eluting before 170°C, eight compounds were tentatively identified by comparison to published mass spectra. These included four aliphatic aldehydes (hexanal, heptanal, nonanal, and undecanal), two alcohols (hexanol and 3-hexenol), one acid (heptanoic acid), and one aromatic aldehyde (phenylacetaldehyde). The aromatic compound was found to have the same retention time and mass spectrum as an authentic sample of phenylacetaldehyde. This compound has been previously isolated from corn silks and found to attract several corn-related pests (Cantelo and Jacobson, 1979). These compounds are presently being bioassayed with field populations of *O. insidiosus*.

This study provides evidence that a synomone attractive to adult *O. insidiosus* probably exists in corn silks. This behavior of *O. insidiosus* may be analogous to the ability of *Podisus maculiventris* (Say) to orient itself toward *Trichoplusia ni* (Hübner) larvae via a chemical stimulus either from the larvae or from a chemical released from injured soybean plants (Greany and Hagen, 1981).

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CARBON-NUTRIENT BALANCE HYPOTHESIS IN
WITHIN-SPECIES PHYTOCHEMICAL VARIATION OF
Salix lasiolepis

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Abstract—Predictions of the carbon–nutrient balance hypothesis were tested using a study of within-species phytochemical variation in the arroyo willow, *Salix lasiolepis*. The prediction that a balance between nutrients (total protein) and carbon-based secondary metabolites (total phenols) should exist was supported using water treatment and fertilizer experiments and wild willow clones. Leaf nitrogen content and net photosynthetic rates of plants potted in soil in which parental plants grew was low, indicating that wild plants exist under relatively low nutrient status–high carbon balance conditions. The hypothesis also correctly predicted positive relationships between shoot length and phenols in glasshouse plants, wild plants, and plants in the water treatment experiment and negative relationships between shoot length and phenols in the fertilizer treatment experiment. Total phenolic glycosides, fragilin, picein, salicortin, tremulacin, and tremuloidin all correlated positively with shoot length in glasshouse plants on a carbon-biased balance, and male willows had generally lower levels of phenolic glycosides than females. Salicortin and tremulacin showed the strongest positive relationships with shoot length.

Key Words—Carbon–nutrient balance, phenolic glycosides, phytochemical variation, salicortin, arroyo willow, *Salix lasiolepis*, shoot length, tremulacin.

INTRODUCTION

The carbon–nutrient balance hypothesis was proposed by Bryant et al. (1983). Much of the hypothesis relates to between-plant species variation in susceptibility to herbivores, but Bryant et al. (1983) also address specifically the anticipated phenotypic responses of plants to nutrient or carbon limitation. For plants with carbon-based secondary compounds like phenols, as in willows, they predict that a balance exists between nutrient concentrations and carbohydrate concentrations in tissues available to herbivores. Under conditions of low nutrients in the soil, but where photosynthesis is not otherwise limited, the carbohydrate-to-nutrient ratio will become higher than in a plant in an unstressed state. Higher carbohydrate concentrations provide a substrate supply for synthesis of carbon-based chemicals such as phenols, which increase in the plant. Lower nutrient concentrations, particularly nitrogen, result in less amino acid and protein synthesis than in the unstressed plant. Therefore, with stress from low nutrients, nutrient content of leaves and shoots declines while carbon-based chemicals increase. The opposite consequence occurs when plants grow in normal nutrient conditions but with a low carbon budget due to shading or low CO₂ concentrations. Nutrient concentrations are high relative to normal carbohydrate concentrations, so nutrient status increases while carbon-based chemicals decline. Because we have been interested in willow variation in relation to herbivore attack, we were interested in testing these predictions with the arroyo willow, *Salix lasiolepis*.

The C/N balance hypothesis also relates to growth rate of a plant. According to Bryant et al. (1983, p. 363) “the decline in growth with nutrient stress is generally greater than the decline in photosynthesis so that carbohydrates and carbon-based secondary metabolites such as phenols accumulate.” Conversely, under carbon stress due to shading, when photosynthesis and carbohydrate concentrations decline “growth rate is reduced more severely than is nutrient absorption, so that tissue nutrient concentrations accumulate above levels necessary to support growth. Under such circumstances one finds a reduction in carbon-based defense such as terpenes and phenolics . . . , because under such circumstances nutrients are cheap relative to carbon.”

We deduce from these arguments that C/N ratios must therefore relate to shoot length, for rapid growth will result in long shoots and slow growth will result in short shoots, both as estimates of mean growth per plant and for examining variation within plants. For example, slower growing shoots on a plant

are likely to be shaded, and therefore grow with a lower C/N ratio than rapidly growing longer shoots in full sunlight. Indeed, based on detailed experiments on C/N ratios in *Salix alaxensis*, Bryant (1987) suggested that shading by alder will increase palatability to snowshoe hares of willow stump sprouts, because carbon-based defenses are reduced in shade. Bryant et al. (1983) also recognized that an individual plant could change in its C/N balance in response to herbivore attack. In addition, in cloning species such as aspen, the same genet maintains a different C/N ratio in juvenile versus mature growth.

One assumption in the carbon-nutrient balance hypothesis, when related to herbivory, is that carbon-based secondary metabolites such as phenols act as defensive compounds against herbivores. A second assumption is that high nutrient status in plants is beneficial to herbivores because of high amino acid and protein concentrations. Some herbivores feed on plant tissues with high phenol concentrations (e.g., Coley, 1983), so the first assumption may not be correct for all herbivores. In general, the second assumption is correct more often than not (e.g., Scriber and Slansky, 1981; Scriber, 1984; Slansky and Scriber, 1985; Mattson and Scriber, 1987). We have addressed these assumptions elsewhere (Waring and Price, 1988), studying total phenolics and total protein in relation to *Salix lasiolepis* and a gall-forming sawfly, *Euura lasiolepis*, finding that neither assumption holds. Our findings do not undermine the basic C/N balance hypothesis, so we concentrate in this paper on the allocational patterns of the willow. However, patterns of chemical allocation in the plant may still affect choice of plants and shoots by *E. lasiolepis* when laying eggs and initiating gall formation, since females appear to use chemical cues on the willow plants. Thus, our ultimate goal remains to understand better the plant-herbivore relationship. We have shown that females attack longer shoots more often than shorter shoots (Craig et al., 1986; Price and Clancy, 1986a), so we were particularly interested in how the carbon-nutrient balance hypothesis related to this pattern.

Salix lasiolepis in northern Arizona grows as a shrub 2–3 m in height and spreads by layering to form clones of one genotype covering about 5–30 m². Clones can be distinguished by such characters as sex, shoot color, and flowering phenology. These clones usually remain distinct also in space because when clones grow toward each other and meet, branches are better supported and layering is prevented. The plant is restricted to such places as springs, artificial ponds, temporary streams, roadside ditches, and other locations that collect and hold water longer than usual in this area of the Colorado Plateau of limestone covered with volcanic lava and ash.

The timing of some sampling of willow coincided with attack by the shoot-galling sawfly, *E. lasiolepis*, which is described in Price and Craig (1984) and Price and Clancy (1986a). The sawfly lays eggs in young shoots and initiates galls, and attack is concentrated primarily in late May and June. The egg hatches

in late June and the larva feeds on gall tissue, spins a cocoon in the gall and overwinters, and emerges from the gall the following spring (Price and Craig, 1984).

In early analyses of phenol and protein concentrations in shoots and leaves of this willow we did not detect clear patterns of variation. We therefore undertook to test the carbon–nutrient balance hypothesis as a possible explanation of varying phytochemicals in relation to shoot length and herbivore attack. To do this we asked the following questions: 1. Does a carbon–nutrient balance occur in *S. lasiolepis*? 2. Which way is the balance tipped in field clones? 3. Do rapidly growing plant parts change in levels of phenols and proteins as types of stress on plants change? 4. Do all phenolic chemicals covary in relation to changes in plant stress?

METHODS AND MATERIALS

In 1982 we surveyed protein and phenol concentrations in young willow shoots. Nineteen willow clones were sampled from an area encompassed by a circle 1 km in diameter located just north of Flagstaff, Arizona, at 2132 m above sea level (35°14'N, 111°30'W). All willows were on the Schultz Creek and Rio de Flag drainages, in and adjacent to Museum of Northern Arizona property. The willow clones were designated according to their local position as follows: Museum of Northern Arizona (MNA, clones 1–7, due east of museum buildings); Northland Press (NP, clones 1–9, near Rt. 180 and 1 km south on Schultz Creek from the MNA site); Coyote Spring (CS, clones 1 and 2) at a small permanently running spring on museum property); and the former Biology Department building of the museum (BD, clone 1). MNA and NP clones on Schultz Creek are on a temporary watercourse, which runs only during spring snow melt, if at all. The BD clone receives much more than normal precipitation from the adjacent building roof. We have retained our original clonal designations to permit comparison with our other papers on this willow (e.g., Price and Clancy, 1986a,b).

For each clone, 10 shoots were sampled on June 9, when shoots were about half grown and while sawflies were attacking shoots (for 1982 phenology of shoot growth and sawfly attack, see Price and Clancy, 1986a). Each sample consisted of a shoot tip 25–30 mm long (including young leaves and associated stem), where sawflies attack and galls form, on shoots that were generally 50–90 mm long. Shoots on the outer perimeter of each clone were sampled, and in addition 10 shoots on NP8 were taken low in the clone where they were shaded. Each shoot sampled was measured for length of shoot. Sample shoots were enclosed in glassine envelopes and kept on ice until stored at –20°C within 1 hr of collection.

Total phenols were determined using the Folin-Denis procedure as described by Zucker (1982) for the samples taken before 1985. The Folin-Ciocalteu phenol reagent has since been found to be better, but the differences are small (Julkunen-Tiitto, 1985). After extraction of phenols, the samples of ground plant were also used to estimate percent total protein. Dried plant material was extracted in 1.0 ml 0.1 N NaOH in a closed tube for 3 hr, stirred for 5 min, and left overnight. Then the samples were stirred for 5 min and centrifuged for 3 min. One-tenth milliliter of supernatant was added to a test tube with 4.9 ml of Bio-Rad protein dye reagent prepared as specified. The optical density (OD) of the sample was measured at 595 nm. Bovine gamma globulin was used as a standard, and its assay was linear over a fivefold range of concentrations. Protein concentrations were expressed as percent protein in dry weight of sample.

In 1982 some potted plants were tested for rate of photosynthesis. For this, juvenile shoot cuttings were taken from wild clones in 1981, rooted in sand, and then transferred to 19-liter pots containing alluvial sandy silt from sites with the parental plants, along Schultz Creek. The clones used were MNA 1, 2, 3, 4; NP 8, 9; and BD 1, with one to three plants representing each clone for a total of 11 plants. In June 1982 these plants were transported to Palo Alto, California, and in September net photosynthesis was measured at light saturation utilizing a system described by Winner and Mooney (1980). Leaf nitrogen of the used leaves was also measured to test for the relationship between leaf nitrogen and net photosynthetic rate to see how these willows compared to other plants in other vegetation types (cf. Field and Mooney, 1986).

In 1982 another set of potted cuttings was started for water and fertilizer treatment experiments in Flagstaff in 1983. Methods were described in Price and Clancy (1986a), so they will be covered only briefly here. For the water experiment, three replicates each of the clones MNA 1, 2; and NP 8, 9 were assigned at random to three water treatments (total of 36 plants). High water (HW) treatment involved saturating each pot with spring water every day through the growing season. The medium water (MW) treatment involved the same treatment once every seventh day. The low water (LW) treatment had water applied in the same way but 22 days apart (May 28 and June 19), and then at 14-day intervals. No fertilizer or chemicals were applied to plants or foliage at any time during 1982 and 1983. For each plant the 10 most distal shoots were measured for length. Five undamaged, young, but fully expanded leaves were taken from two plants per clone per treatment (total of 120 samples) on August 17, 1983, and treated as above for total phenol and percent protein analyses. The positive effects of increased water on mean shoot length and sawfly attack have been reported (Price and Clancy, 1986a).

For the fertilizer experiment three or four replicates each of the clones MNA 1, 2, 3, 4 were assigned at random to three fertilizer treatments (total of

39 plants). All pots received the high water treatment as above. The no fertilizer treatment (HW/OF) had only water applied. The low fertilizer treatment (HW/LF) received fertilizer once per week, and the high fertilizer treatment (HW/HF) had fertilizer added twice per week, both for the period of maximum willow growth rate from May 28 to June 27, 1983. A fertilizer application consisted of 6.2 g Miracle Gro dissolved in 1 liter of water (15% N, 30% P, 15% K general fertilizer), with solution added to saturate the pot. Five leaves per plant on three plants per clone per treatment (180 samples total) were collected as in the water experiment for total phenol and percent protein analyses.

To study relationships between shoot length and individual phenol glycosides, five plants per clone on six clones were rooted in sand in the greenhouse in Flagstaff for three weeks in 1986. Three pairs of male and female clones were used as donors for cuttings, each pair growing within a few meters in the field: MNA 2 male, MNA 6 female; CS 2 male, CS 1 female; and NP 8 male, NP 9 female. They were transported as washed-root cuttings to Joensuu, Finland (62°30'N, 29°50'E) and, using potting soil (85% pure sand and 15% peat), grown in 3.6-liter pots in the glasshouse from May 19. On June 27, shoot samples were taken for phenol glycoside analysis. Up to 10 of the longest shoots per clone and up to 10 of the shortest shoots per clone were sampled, with at least five of each per clone. Shoot lengths were measured, and then tips 25–30 mm long were removed for analysis. Shoot tips were treated individually for GLC analysis. Briefly, samples were extracted in 80% acetone, filtrated, concentrated, and dissolved into methanol. The sample derivation and OV-1 capillary column analysis were processed as described in Julkunen-Tiitto (1985). From these analyses, individual phenol glycoside concentrations were calculated as milligrams per gram dry weight of plant. Lindroth and Pajutee (1987) have criticized the methods of Julkunen-Tiitto (1985), who used oven-dried samples for extraction. However, in response, Julkunen-Tiitto and Tahvanainen (1988) showed that there was a perfect correlation between individual phenol glycoside concentrations when extracted from fresh and oven-dried leaves ($N = 5$, $r^2 = 1.00$, $P < 0.01$). Therefore, we conclude that the methods used in the present paper, which were those employed by Julkunen-Tiitto and Tahvanainen (1988), are valid. Lindroth and Pajutee (1987) showed high and significant correlations between fresh and oven-dried samples in phenol glycoside concentrations in four of five species, with only *Populus tremuloides* samples showing radical differences. Thus these results and those of Julkunen-Tiitto and Tahvanainen (1989) show little discrepancy for willows.

Woody plants are heterogeneous in many characters. Watson and Casper (1984) speak of plants as assemblages of semiautonomous physiological units. Pitelka and Ashmun (1985) concluded that local vascular integration is more common than integration throughout the whole genet. Modular development and control in plants provides "relative autonomy among plant parts" (Waller,

1986, p. 298). Zucker (1982) recorded impressive differences in total phenol concentrations between leaves on the same poplar trees. Variation within plants to herbivore attack is well documented (Denno and McClure, 1983; Whitham et al., 1984; Gill, 1986). Therefore, modules such as shoots are likely to have individualistic properties, independent from all but the closest adjacent modules. For this reason we use individual shoots as samples in some analyses, because we have no evidence to show that they are not responding independently in the variables we have measured.

RESULTS

Carbon-Nutrient Balance. Tests among wild clones and experimental plants all shared a negative relationship between total phenols and total proteins, as predicted by the carbon-nutrient balance hypothesis (Table 1). In the fertilizer experiment nutrient status was increased, phenols were depressed, and the negative relationship was most clearly seen, accounting for 77% of the variance. Individual samples were tested to see if the balance operated within leaves and shoot tips, and in all cases a significant negative relationship was found. The only exception to this general pattern was the within-clone variation in NP 8 where the relationship was not significant and weakly positive.

Balance in the Field. A strong and positive relationship existed between leaf nitrogen and net photosynthesis, and the regression equation accounted for 83% of the variance (Figure 1). These nitrogen and photosynthesis levels were

TABLE 1. RELATIONSHIPS BETWEEN TOTAL PHENOL CONCENTRATIONS (OD 725/mg DRY WT) (X) AND TOTAL PROTEIN CONCENTRATIONS (% DRY WT) (Y) IN POTTED EXPERIMENTAL PLANTS AND IN WILD CLONES

Sample source	Equation	Sample size	r^2	Probability
1983 Water experiment (2 leaves/plant)				
Per clone per treatment	$Y = 12.46 - 4.82X$	12	0.17	N.S.
Per individual leaf	$Y = 12.31 - 4.51X$	24	0.17	<0.05
1983 Fertilizer experiment (3 leaves/plant, except 2 clones with 2 leaves/plant)				
Per clone per treatment	$Y = 17.35 - 17.12X$	12	0.77	<0.01
Per individual leaf	$Y = 16.72 - 15.15X$	34	0.37	<0.01
1982 Wild clones (9-10 shoot tips/plant, except NP 8 with 20 shoot tips)				
Per clone	$Y = 37.27 - 15.81X$	19	0.09	N.S.
Per individual shoot tip	$Y = 30.93 - 8.17X$	195	0.06	<0.01
1982 Within wild clone (NP 8)	$Y = 22.96 + 0.09X$	20	0.00005	N.S.

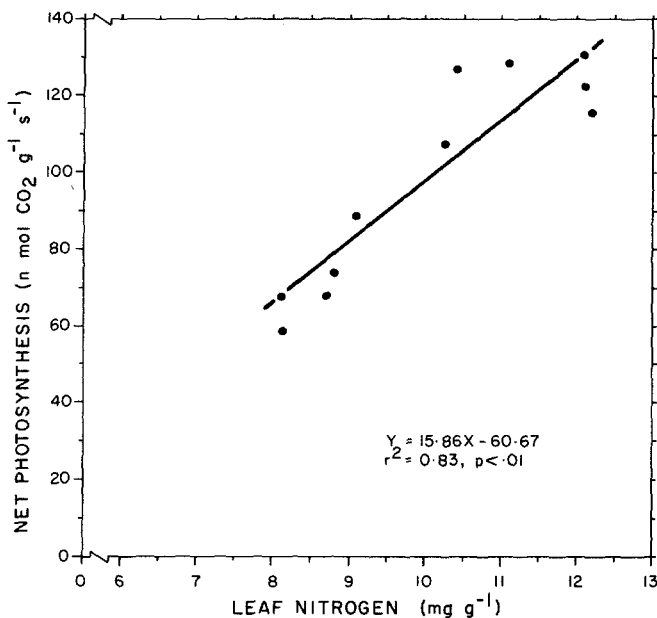


FIG. 1. Relationship between leaf nitrogen (mg/g) and net photosynthesis (nmol CO₂/g/sec) in leaves of willow plants potted in soil in which the parental clones grow.

comparable to plants growing in low-nutrient habitats, such as chaparral and fynbos plants in South Africa (see Field and Mooney, 1986). These levels are among the lowest recorded in a wide range of vegetation types, clearly indicating low-nutrient status soils, as to be expected in alluvial sandy silt deposited by a mountain stream. The willows growing in these soils, mostly in full sun or slight shade for some of the day, will have low-nutrient status, and the balance will be shifted to a high-carbon-low-nutrient condition.

Balance Shift with Stress Changes. Plants growing with a high-carbon-low-nutrient balance should show increasing levels of carbon-based secondary metabolites as shoot length increases, according to the hypothesis and Bryant's (1987) empirical results. In every case tested, where plants were grown with a relatively positive carbon balance, there was a significant and positive relationship between phenols and shoot length (Table 2). This was true for phenol glycoside content of willows grown in the glasshouse in Finland in 1986, both in male and female plants, and for total phenols in water treatment experimental plants in 1983, and in wild plants in 1982. In general, female willows had phenol glycoside concentrations double that of males (intercept for females 20.34 mg/g dry wt, for males 9.49, with similar slopes, 0.21 and 0.26, respectively).

TABLE 2. RELATIONSHIPS BETWEEN SHOOT LENGTH (X) AND TOTAL PHENOL GLYCOSIDE (mg/g DRY WT) AND PHENOLS (OD 725/mg DRY WT) CONCENTRATIONS (Y) IN SHOOTS IN POTTED PLANTS GROWN IN THE GLASSHOUSE, FIELD, AND WILD CLONES

Sample source	Equation	Sample size	r^2	Probability
1986 Glasshouse all plants ^a	$Y = 14.04 + 0.24X$	12	0.73	<0.01
Males	$Y = 9.49 + 0.26X$	6	0.71	<0.05
Females	$Y = 20.34 + 0.21X$	6	0.76	<0.05
1983 Water experiment ^b	$Y = 0.27 + 0.0042X$	24	0.21	<0.05
1982 Wild plants	$Y = 0.14 + 0.01X$	19	0.29	<0.05
1983 Fertilizer experiment	$Y = 0.46 - 0.00077X$	22	0.17	0.05 < P < 0.10
1982 Within wild clone (NP8)	$Y = 0.87 - 0.0044X$	20	0.44	<0.01

^aTotal phenol glycosides were measured in 1986.

^bTotal phenolics were measured in 1982 and 1983.

When we relieved the nutrient stress in the fertilizer experiment in 1983, nutrient status increased and phenols declined in relation to shoot length, as the hypothesis predicted, producing a negative relationship between shoot length and total phenol concentrations (Table 2). Within the wild clone NP 8 in 1982 the shoots low in the canopy were shaded but grew longer because they were on younger branches (cf. Craig et al., 1986), so they grew under carbon stress relative to the shorter shoots in full sunlight. So the longer shoots should have a lower concentration of phenols than shorter shoots, producing a negative relationship between shoot length and phenol concentration, as we found in this clone (Table 2).

Covariance of Phenol Glycosides. In plants grown in the glasshouse in Finland under very long daylight conditions at 62°N in June (daylight peaking at about 22 hr per day), there was a strong positive relationship between total phenolic glycosides and shoot length, accounting for 73% of the variance (Table 3). Five of the individual phenol glycosides, fragilin, picein, salicortin, tremulacin, and tremuloidin, showed the same pattern. Only salicin varied independently of shoot length, but generally constituted a small part of the total phenol glycoside content (Table 4). The chemical structures of all compounds are illustrated in Meier et al. (1985) and Lindroth et al. (1987). Salicortin and tremulacin were the major phenol glycosides present. They were the most strongly related in concentration to shoot length in terms of a strong slope in relation to shoot length, with 83% and 59% of the variance accounted for (Tables 3 and 4).

It is interesting to note that male plants had a carbon balance different from females (Table 4), at least in relation to phenol glycoside concentrations (see

TABLE 3. RELATIONSHIPS BETWEEN MEAN SHOOT LENGTH PER CLONE SAMPLE (mm) (X) AND PHENOLIC GLYCOSIDE CONTENT OF SHOOT TIPS (mg/g DRY WT) (Y) ON PLANTS GROWN IN THE GREENHOUSE (SAMPLE SIZE WAS 12 FOR EACH REGRESSION)

Phenolic glycoside	Equation	r^2	Probability
Total phenolic glycosides ^a	$Y = 14.04 + 0.24X$	0.73	<0.01
Fragilin	$Y = 0.40 + 0.01X$	0.75	<0.01
Picein	$Y = 0.24 + 0.01X$	0.54	<0.01
Salicin	$Y = 1.75 + 0.002X$	0.03	N.S.
Salicortin ^a	$Y = 6.01 + 0.11X$	0.83	<0.01
Tremulacin ^a	$Y = 4.74 + 0.12X$	0.59	<0.01
Tremuloidin	$Y = 0.90 + 0.01X$	0.51	<0.01

^aRelationships with large increases as shoot length increased.

also Table 2). In many of the pairwise comparisons of males and females that grow in proximity in the field, for both long shoot and short shoot comparisons, males had lower levels than females in total phenol glycosides, and the constituent glycosides (males were invariably lower than females in total phenol glycosides, salicortin and tremuloidin for both short and long shoots). Thieme (1965) and Palo (1984) noted such differences in the bark of some other willow species, with *Salix caprea* showing the largest sexual difference: females had almost two times the concentration of males. Such differences could contribute to the observed female-biased sex ratios in willows and preference for feeding on males by voles (e.g., Danell et al., 1985).

DISCUSSION

We have shown that predictions from the carbon-nutrient balance hypothesis, relating to the within-species variation of arroyo willow, fit the data to a remarkable degree. Every significant relationship we found was explained adequately by the hypothesis. We know of no alternative hypothesis that could account for so much of the variation seen in this willow species.

Although we always tested several willow genotypes, they all fitted the general patterns detected, suggesting that the environments in which the willows grow, and consequent shoot length, play a large part in phenotypic variation. For example, in the strongest relationships, such as in the glasshouse plants, shoot length accounted for 71–76% of the variance in total phenol glycoside content (Table 2), and we know that shoot length is strongly influenced by such nongenetic traits as plant age (Craig et al., 1986), and nutrient and

TABLE 4. PHENOLIC GLYCOSIDE CONTENT (mg/g DRY WT) OF LONG AND SHORT SHOOTS ON SIX WILLOW CLONES GROWN IN THE GLASSHOUSE (MEANS ARE FROM 3-6 SAMPLES PER SHOOT LENGTH CLASS PER CLONE)

Clone	Sex	Shoot length class	Mean shoot length (mm)	Phenolic glycoside						
				Total	Fragilin	Picein	Salicin	Salicortin	Tremulacin	Tremuloidin
MNA 2	Male	Short	35	21.1	0.4	0.8	2.1	8.7	7.9	1.2
MNA 6	Female	Short	53	28.9	0.6	0.3	2.8	11.9	11.4	1.9
CS 2	Male	Short	44	17.3	0.5	0.1	1.1	8.7	6.2	0.7
CS 1	Female	Short	47	38.8	1.0	0.9	1.9	17.2	16.2	1.6
NP 8	Male	Short	51	21.6	0.8	0.4	1.1	9.2	8.9	1.2
NP 9	Female	Short	46	23.5	0.7	0.7	1.2	10.7	8.9	1.3
MNA 2	Male	Long	89	41.5	1.2	1.7	1.6	14.9	19.6	2.5
MNA 6	Female	Long	172	61.3	1.5	1.7	1.7	24.6	28.6	3.2
CS 2	Male	Long	122	30.1	1.2	0.7	1.2	15.9	9.6	1.5
CS 1	Female	Long	139	55.0	1.5	1.3	1.6	23.9	24.6	2.1
NP 8	Male	Long	137	51.7	1.4	1.3	1.5	19.7	26.1	1.7
NP 9	Female	Long	161	45.1	1.1	1.4	1.4	22.9	16.2	2.1

water status of the environment (Price and Clancy, 1986a). This leaves very little variation to be accounted for by genotypically determined traits.

We must conclude that willows growing in natural conditions will be profoundly influenced by environmental variables, such as water supply, soil nutrients, and shading, in terms of their carbon-nutrient balance, and genotypic variation may be small. Several relationships between the factors measured, such as phenol-protein concentrations and shoot length-phenol correlations, changed between positive and negative slopes as water, nutrients, and shading varied.

The balance between nutrients and secondary metabolites, which are thought to play a defensive function, has been recorded in many other within-plant species systems. Leaf nitrogen was inversely related to tannin content (Jones, 1976; Phillips and Henshaw, 1979; Mattson, 1980). High protein concentrations reduced toxicity of phenolic compounds (Kirkham, 1954; Kiraly, 1976; Duffey et al., 1986), and proteins and phenolics were inversely correlated in *Salix lasiolepis* (Waring and Price, 1988). An inverse relationship between nitrogen content and glucosinolate levels in crucifers has also been described (Joseffson, 1970; Wolfson, 1980). Although Rhoades (1979) does not address the carbon and nutrient balance directly, emphasizing only responses to stress in plants in terms of "defensive" chemicals, three or four examples, out of seven in woody plants, are cited in which shading is correlated with decreases in tannins, which may be regarded as carbon-based defenses (his Table 2). Mattson (1980, p. 138) is emphatic that "Low plant N concentrations are invariably associated with . . . high allelochemic contents," citing three cases of increased N with decreased phenolics and five cases of decreased N with increased phenolics. A recent additional case is provided by Mihaliak et al. (1987).

What does the variation in phenols and proteins mean for the *S. lasiolepis* herbivore *E. lasiolepis*? We know that females attack independently of protein and total phenols in experiments (Waring and Price, 1988). However, in this isolated population of *S. lasiolepis*, it is probable that the large majority of plants are nutrient stressed, so that phenolic levels correlate with shoot length. Since females attack longer shoots more frequently (Craig et al., 1986, Price and Clancy, 1986a), they may be responding to total phenolic glycosides or individual phenolic glycosides (cf. Tables 3 and 4), in which case salicortin and tremulacin would provide the strongest indicators of shoot length. These compounds are actively defensive in *Populus tremuloides* against *Papilio glaucus glaucus* (Lindroth et al., 1988). Furthermore, female sawflies avoid previous oviposition wounds made by other females based on chemical cues, which could involve oxidation products of phenol glycosides (Craig et al., 1988). Therefore, levels of phenolics in shoots may relate to the levels of discrimination against

oviposition sites by subsequent female sawflies. These possibilities have yet to be tested.

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ACQUIRED CHEMICAL DEFENSE IN THE LYCAENID BUTTERFLY, *Eumaeus atala*

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Abstract—The lycaenid butterfly, *Eumaeus atala*, was found to contain cycasin, an azoxyglycoside, by thin-layer chromatography (TLC). Quantification of cycasin content in 10 individual freshly killed and frozen males and females, using capillary gas chromatography (GC), showed that cycasin contents of individual butterflies ranged from 0.21 to 0.51 mg (1.24–2.75% dry weight). A museum specimen of *E. atala* of unknown age had undetectable amounts of cycasin by GC. GC showed that larval frass contained about 0.10% cycasin, which was not detectable by TLC. Cycasin in the host plant was not detectable by TLC but was detected by GC and found to be 0.02% dry weight. There was no macrozamin, another azoxyglycoside characteristic of many cycads, in the butterfly or plant. Feeding trials with a colony of the ant, *Camponotus abdominalis floridanus*, showed that both cycasin and the adult of *E. atala* were deterrent to the ants.

Key Words—Cycasin, macrozamin, cycads, azoxyglycosides, *Eumaeus atala*, Lepidoptera, Lycaenidae, insect-plant interaction, chemical ecology, unpalatability, insect defense strategy, ants, *Camponotus abdominalis floridanus*.

INTRODUCTION

A variety of butterfly species in several families are known to be unpalatable to predators, including members of the Papilionidae, Pieridae, and Nymphalidae (e.g., Brower, 1984; Rothschild, 1985). In many of these species, unpalatable and/or toxic qualities are due to compounds sequestered from the larval

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hostplant (e.g., Brower, 1984; Rothschild, 1985). Representatives of the Lycaenidae are conspicuously absent from this list and are not known to be unpalatable or toxic to potential predators, although Eliot (1973) suggests that members of the *Danis* section of the Polyommataini are unpalatable. Instead, lycaenids appear to use crypsis, false heads (Robbins, 1980, 1981), and associated ants (Eliot, 1973; Atsatt, 1981; Pierce, 1983) as defenses. The diversity of form and coloration among the lycaenids is striking (see Seitz, 1924; Howe, 1975), ranging from cryptic to brilliant warning coloration.

The genus *Eumaeus* Hübner is notable among lycaenids for the bright coloration of adults and larvae. In general, the adults are black with iridescent blue-green on the wings, and a bright red abdomen. The exception to this is *E. childrenae* Gray, which does not have a red abdomen. The gregarious larvae are bright red with yellow tubercles. Interestingly, the pupae are also warningly colored red-orange and are often found conspicuously clumped (DeVries, 1977) in contrast to other species of lepidopteran pupae, which are cryptic and solitary (Brower, 1984). The larvae of *Eumaeus* feed on cycads in the genera *Xamia* and *Cycas* (Cycadaceae). Cycads are known to contain a group of unusual nitrogenous compounds, azoxyglycosides, the aglycones of which are carcinogenic and mutagenic and are responsible for livestock poisoning in some areas of the world (Hooper, 1978). Two of the best characterized of these compounds are cycasin and macrozamin (Figure 1) (de Luca et al., 1980; Moretti et al., 1983). DeVries (1976, 1977) suggested that *Eumaeus minyas* Hübner was unpalatable, based on larval and adult coloration and larval host-plant alliances. Work by Teas (1967; Teas et al., 1966) showed that larvae of the warningly colored moth, *Seirarctia echo* Abbott and Smith (Arctiidae), accumulated cycasin after feeding on cycad leaves. Rothschild et al. (1986) found that one species of *Eumaeus*, *E. atala floridana* (Poey), contained cycasin. Here, we confirm this report and compare capillary gas chromatography (GC) and thin-layer chro-

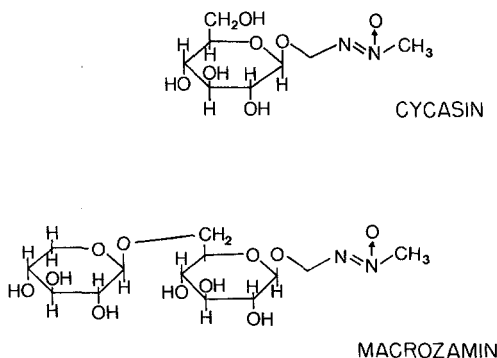


FIG. 1. The structures of cycasin and macrozamin.

matography (TLC) as methods for detecting cycasin. Feeding experiments with ants and birds show that *E. atala* is unpalatable to representative vertebrate and invertebrate predators.

METHODS AND MATERIALS

Butterflies and Plants. *Eumaeus atala* was thought to be extinct in the mainland United States for about the past 35 years (Rawson, 1961; Lenczewski, 1980). Recently, several populations were discovered in the Miami area of Florida (Lenczewski, 1980), and it is now relatively common in many areas of southern Florida (Robbins, personal communication). Whether these populations resulted from small, scattered, mainland colonies that had remained undiscovered or from colonizers from Cuba or the Bahamas is unknown. *Eumaeus atala* had not been abundant in the Miami area since the 1920s, although a colony was known from Key Biscayne in 1961 (Rawson, 1961; Lenczewski, 1980). The host plant of *E. atala* in Florida is a cycad called coontie, *Xamia floridana* A. DC.

Eggs of *E. atala* were obtained in the course of a mark-release-recapture study in the Miami area (J. Weintraub, unpublished data). Larvae were reared in a growth chamber, first on *X. floridana* leaves brought from Florida and, when these leaves were eaten, leaves were obtained from greenhouse grown specimens of *X. floridana*. *E. atala* larvae were reared in plastic boxes under conditions of 14:10 light-dark, 30°C day:25°C night.

Butterflies were frozen upon emergence and kept in the freezer until prepared for GC or TLC. Butterflies were dried at 50°C for two days prior to analysis.

To determine whether azoxyglycosides could be detected in museum specimens, a specimen of *E. atala* of unknown age and lacking data, from the collection of the Museum of Comparative Zoology, Harvard University, was also analyzed.

Plant material for chemical analysis was obtained from greenhouse specimens of *X. floridana*.

Preparation of Butterfly and Plant Samples for Thin-layer Chromatography. Butterfly samples for TLC were prepared using a modification of the procedure of Yagi et al. (1980). They were first ground with 100 mg sand in a mortar. Then, samples were suspended in ice-cold 7:3 ethanol-water (10–15 ml), and debris was precipitated by centrifugation at 2000 rpm. The precipitate was washed three or four times with 2–5 ml ice-cold 7:3 ethanol-water, centrifuged at 2000 rpm after each washing, and the supernatants combined. These combined supernatants were evaporated to about 500 μ l under vacuum at 40°C. The whole butterfly sample was composed of two butterflies with a combined

weight of 60 mg. The butterflies were not sexed. The butterfly parts were obtained from three different butterflies; a total of 21.9 mg wings, 31.5 mg abdomen, and 33.9 mg head + thorax were analyzed.

Larval frass was collected during larval feeding on *X. floridana*, dried, and stored in the freezer prior to analysis. Larval frass (563 mg) was ground with 550 mg sand and extracted with cold ethanol as described for the butterflies.

Two methods were used to extract the plants. The first was that used for the butterflies. A second sample was prepared using leaves frozen in liquid nitrogen, which were then extracted as described above for insect samples.

Thin-layer Chromatography. Samples of 8 μ l of the total sample were spotted onto glass-backed silica gel TLC plates that were developed in a solvent system of *n*-butanol-acetic acid-water 4:1:1 (Matsumoto and Strong, 1963). Plates were sprayed with chromotropic acid reagent, composed of 1 g of 4,5-dihydroxy-2,7-naphthalene disulfonic acid disodium salt dissolved in 100 ml water and diluted to 500 ml with 12.5 M sulfuric acid (Matsumoto and Strong, 1963) and then heated at 110°C for 10–15 min.

Standards of purified cycasin and macrozamin were obtained from Dr. K. Tadera (Kagoshima University). Standard solutions were made up in 7:3 EtOH-H₂O, at 1 mg/ml for cycasin and 2 mg/ml for macrozamin.

The presence of cycasin in butterfly and plant samples was confirmed by the comigration of standards and butterfly and plant samples, spotted separately. Standards were also mixed with the samples and run on TLC. In addition, we confirmed the presence of cycasin in the samples by observing the disappearance of cycasin following incubation of another aliquot of the butterfly and plant solutions with β -glucosidase (Sigma). β -Glucosidase was used at a concentration of 1 mg/ml and was added to samples in 25 mM NaOAc (pH 5.0), and incubated at 30°C for 18 hr.

Preparation of Samples for Gas Chromatography. Gas chromatography was used to corroborate and quantify the presence or absence of cycasin in *E. atala* butterflies, larval frass, and *X. floridana* leaves. Ten *E. atala* butterflies, four females and six males, were crushed individually and extracted 3 \times in 5.0 ml 80% methanol in water (Rothschild et al., 1986). One female was analyzed whole, and the other nine butterflies were analyzed as abdomens separate from head, thorax, and wings. This technique was used to determine whether we would be able to assay the abdomens of specimens as an indication of the specimen's total cycasin content. In addition, the museum specimen was analyzed as abdomen separate from the wings + head + thorax. The samples were filtered and the filtrate evaporated to dryness. To this residue, 1.0 ml of catalpol (0.75 mg/ml water) was added as an internal standard, and the mixture evaporated to dryness. To this mixture, 1.0 ml MeOH was added and 100 μ l removed to a small glass sample tube and evaporated to dryness. The sample was sily-

lated by adding 0.10 ml of the silylation reagent TRI-SIL Z (Pierce Chemical Company) and the closed tube heated for 15 min at 70–80°C. After the solution was cooled, 1 μ l was injected onto the gas chromatograph. A solution of cycasin (0.50 mg/ml) and catalpol (0.75 mg/ml water) was silylated and used as a standard.

The dried frass sample (446 mg) was prepared for analysis using the same methods as for the butterflies. Plant material was extracted fresh (10.73 g wet weight), using the method of Rothschild et al. (1986).

The GC analyses were performed on a Hewlett Packard 5890 gas chromatograph equipped with a split-splitless injector, FID detector, and a 3393 Hewlett Packard integrator. The column was a DB-1 capillary (J&W Scientific), 30 m long and 0.32 mm ID, with a film thickness of 0.10 μ m. The injector was used in the split mode, split ratio of 13:1, and at a temperature of 275°C. Carrier gas was helium. The detector temperature was 325°C. Samples were run under the following program developed by Gardner (1987) for iridoid glycosides: initial column temperature 200°C for 1.00 min, which was then raised to 260°C at a rate of 20°/min and held for 6.00 min. The retention time (T_r) of cycasin was 4.05–4.07 min, of macrozamin 7.11 min, and of catalpol 7.95–8.00 min.

Feeding Experiments with Ants. To determine whether *E. atala* was unpalatable and whether cycasin was important in the chemical defense of *E. atala*, we used a colony of *Camponotus abdominalis floridanus* (Buckley) ants reared from a founding queen collected on Saddlebunch Key, Monroe County, Florida, in 1983, as potential predators. Although there are no published data on the natural diet of this species, they appear to be general predators and have been observed capturing scorpions and beetles in their natural habitat (N. Carlin, personal communication). This colony contained about 60–100 workers and soldiers and a queen, and was housed in a 20 \times 30-cm plastic box. The ants nested in glass test tubes and foraged in the clear area of the box. Ants were maintained on a diet of crickets and honey water.

Three experiments with the ants, using techniques similar to those of Pasteels et al. (1986), were used to determine whether cycasin was an effective chemical defense against such predators. Ants were starved for 48 hr prior to all the experiments, so that they would forage consistently during the testing.

In the first experiment, the ants were offered large drops of two solutions. The control solution was 25% sucrose, and the experimental solution was 25% sucrose + 1.0 mg cycasin/ml water. Ants were allowed to forage at these two drops for an hour. Additional solution was added when necessary. Every minute, the number of ants that visited and drank at the solution was tallied. An ant was considered to be drinking when it lowered its head and contacted the solution. With magnification, the ant's tongue could be observed in the solu-

tion. This experiment was repeated for a second hour the following day. In addition, in three separate trials, the length of time that the ants drank at each of these solutions was recorded.

A second experiment compared the behavior of the ants in response to a drop of a 25% sucrose solution versus a dried, ground *E. atala* butterfly in 25% sucrose solution (8.5 mg/ml). The number of ants drinking from the two drops was again monitored every minute over the course of an hour. This experiment was also repeated a second time, one week after the first trial.

A third experiment allowed ants to forage at two drops of water, the control containing ground up cricket, and the other containing ground up *E. atala* butterfly. The number of ants drinking at each drop was again counted every minute for an hour. This was repeated a second and a third time. The second trial was 22 days after the first and the third trial was 10 days later.

RESULTS

Chemical Analyses. TLC indicated that *E. atala* butterflies contained a compound with the same R_f as cycasin: in the standard the R_f was 0.32, and in the butterfly it was 0.31. Cycasin was present in the whole butterflies as well as in the wings, head + thorax, and the abdomen. The R_f of macrozamin was 0.19, and there was none detectable in the butterflies. There was no cycasin or macrozamin detectable by TLC in the larval frass. We were unable to detect cycasin in the leaves of *X. floridana* by TLC, as Teas (1967) had also found. Rothschild et al. (1986), however, did find cycasin in leaves of *X. floridana* using gas chromatography, which we did as well.

GC analyses showed that cycasin (R_t 4.05–4.07 min) occurred in relatively large amounts in both male and female *E. atala* butterflies (Table 1). For both males and females, the amounts in the abdomens were lower than those in the wings + head + thorax (Table 1). There was no detectable cycasin in the abdomen or wings + head + thorax of the museum specimen. No peak with the same R_t as macrozamin (7.11 min) was detectable in the butterflies by GC.

To determine whether the amount of cycasin in the abdomen was indicative of the amount in the whole butterfly, we attempted to correlate cycasin content of abdomen and the rest of the butterfly, using the data from the nine frozen individuals analyzed in parts. A plot of dry weight of the abdomen versus dry weight of the wings + head + thorax showed that these were highly correlated (Figure 2A). A similar plot of percent cycasin in abdomen versus percent cycasin in wings + head + thorax also showed a significant positive correlation (Figure 2B). Thus, analyzing only the abdomen indicated reliably whether the individual contained cycasin and could give a good indication of how much that individual contained.

TABLE 1. CYCASIN CONTENT OF MALE AND FEMALE *Eumaeus atala* BUTTERFLIES AS DETERMINED BY GAS CHROMATOGRAPHY

	N	Dry weight (mg, mean \pm SD)	Range	Cycasin content (mg, mean \pm SD)	Range	Cycasin (%, mean \pm SD)	Range
Males							
Abdomen	6	7.28 \pm 2.76	5.13-12.66	0.04 \pm 0.02	0.01-0.07	0.60 \pm 0.23	0.21-0.91
Wings + head + thorax	6	14.20 \pm 2.37	11.19-30.21	0.32 \pm 0.10	0.20-0.44	2.20 \pm 0.44	1.81-2.61
Total	6	21.48 \pm 4.97	16.32-30.21	0.36 \pm 0.12	0.21-0.51	1.66 \pm 0.33	1.24-2.06
Females							
Abdomen	3	5.51 \pm 0.69	5.04-6.31	0.06 \pm 0.03	0.03-0.08	1.08 \pm 0.57	0.43-1.50
Wings + head + thorax	3	10.78 \pm 0.95	9.80-11.70	0.30 \pm 0.09	0.22-0.40	2.75 \pm 0.60	2.19-3.38
Whole butterfly	1	21.59		0.41		1.90	
Total	4	17.68 \pm 2.67	16.04-21.59	0.37 \pm 0.09	0.24-0.46	2.11 \pm 0.54	1.50-2.75

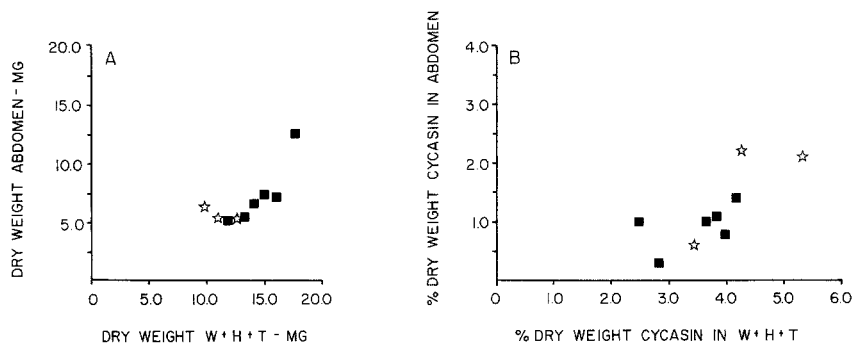


FIG. 2. Dry weight (A) and cycasin content (B) of *E. atala* abdomen versus wings + head + thorax. open stars = females, solid squares = males. Correlations: (A) $r = 0.801$, $p = 0.01$; (B) $r = 0.710$, $p = 0.03$.

GC also showed that the frass sample contained about 0.1% dry weight of cycasin. Interestingly, the frass sample contained a peak with the same retention time (7.11 min) as macrozamin. Since no such peak occurred in the plant or butterflies analyzed, if this peak is indeed macrozamin, it suggests that the larvae may be converting something from the plant into macrozamin. Alternatively, some *X. floridana* plants may contain macrozamin. Larvae of *E. atala* were collected in Florida, and we were unable to analyze plant material from there.

According to GC analyses, the *X. floridana* frond contained about 0.02% dry weight cycasin.

Feeding Experiments with Ants. In both feeding trials of cycasin versus sucrose, the ant foragers initially fed at both solutions, but after 10–20 min, they stopped drinking at the cycasin solution and continued to drink at the sucrose solution (Figure 3). They did revisit the cycasin, but only briefly. A comparison of the total number of visits to the two solutions showed that significantly more ants visited the sucrose solution in both trials (trial 1, expected = 32.5, $\chi^2 = 11.22$, $P < 0.005$; trial 2, expected = 31, $\chi^2 = 18.65$, $P < 0.001$). The ants did not exhibit any apparent alteration in behavior or any toxic responses from drinking the cycasin solution. The decrease in visits to the cycasin solution while the visits to the sucrose solution remained relatively high suggests that the ants learned to avoid the cycasin solution. On the second trial on the following day, however, the ants went through the same process of initially drinking from both solutions and then avoiding the cycasin solution (Figure 2). Despite this behavior, the amount of time that the ants spent drinking at the two solutions was not significantly different (one-way ANOVA, $df = 1.65$; $F = 3.27$; $0.05 < P < 0.10$) (Figure 4).

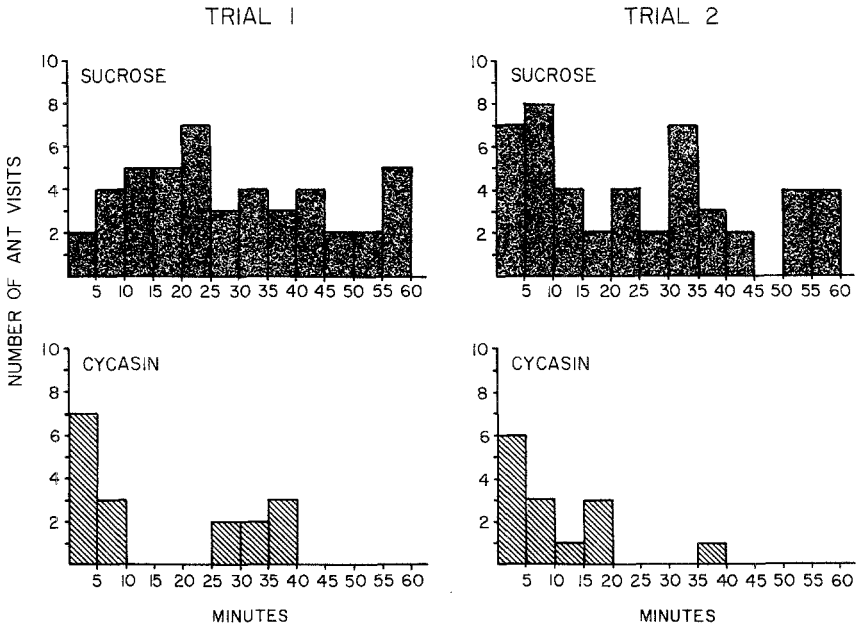


FIG. 3. The response of foraging *C. abdominalis floridanus* ants to a choice of drops of solutions of 25% sucrose and 25% sucrose + 1.0 mg/ml cycasin. The number of ants drinking from the drops was recorded every minute, but the data were combined for 5-min intervals for presentation.

The ants showed a similar response to the *E. atala* in 25% sucrose. These experiments were performed later in the spring than the previous experiments (March for the first set and June for the second), and the colony had increased in size from about 60 to about 100. In this experiment, there were many more ants foraging at both drops initially; as many as 12 ants were drinking at the drops at the same time. The ants initially fed equally often from the control drop and the drop containing the ground up butterfly in the first trial, but in the second trial fewer ants initially visited the drop containing *E. atala* (Figure 5). The total number of ant visits was significantly higher to the sucrose solution in both trials (trial 1, expected = 99, $\chi^2 = 16.99$, $P < 0.005$; trial 2, expected = 81, $\chi^2 = 43.56$, $P < 0.001$).

The results from the trials with the cricket versus the ground *E. atala* butterfly in water showed that the ants avoided the water solution containing the *E. atala* butterfly (Table 2). The number of ant visits in this experiment was one quarter to one sixth that of the experiments using sucrose solutions, probably because sucrose is more attractive than protein.

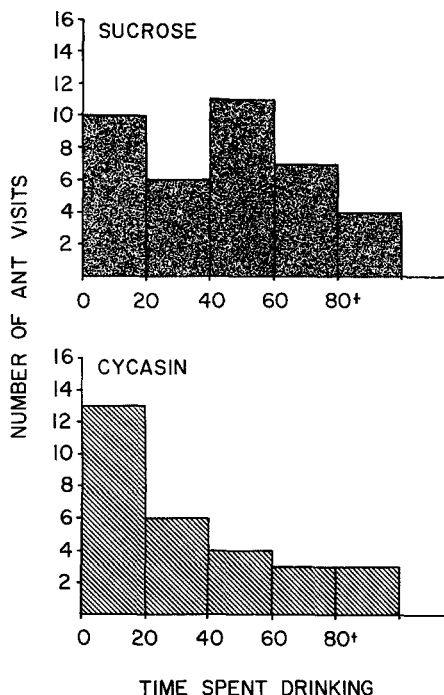


FIG. 4. The lengths of time that *C. abdominalis floridanus* ants spent drinking at solutions of 25% sucrose and 25% sucrose + 1.0 mg/ml cycasin. Both drops were available to the ants at the same time.

DISCUSSION

The warning coloration of larvae, pupae, and adults of *E. atala*, and other *Eumaeus* species is striking. In addition, all life stages of *E. atala* and *E. minyas* (= *E. toxea*) (DeVries, 1976), and probably other *Eumaeus*, are gregarious. These characteristics, typical of unpalatable insects, suggested that *E. atala* and the other species of *Eumaeus* are unpalatable (DeVries, 1977). Our results showed that *E. atala* butterflies contain cycasin in amounts as high as 2.75% dry weight. Preliminary results of GC analyses of *E. toxea* and *E. goddardii* indicate that they contain cycasin as well (Bowers, Collinge, and Robbins, unpublished data). In addition, ants visited solutions of 25% sucrose or water containing *E. atala*, as well as a solution of 25% sucrose containing cycasin, significantly less than control solutions of 25% sucrose or water with ground cricket, suggesting that *E. atala* and cycasin itself are unpalatable to these ants.

Feeding trials with gray jays (*Perisoreus canadensis*, Corvidae) showed

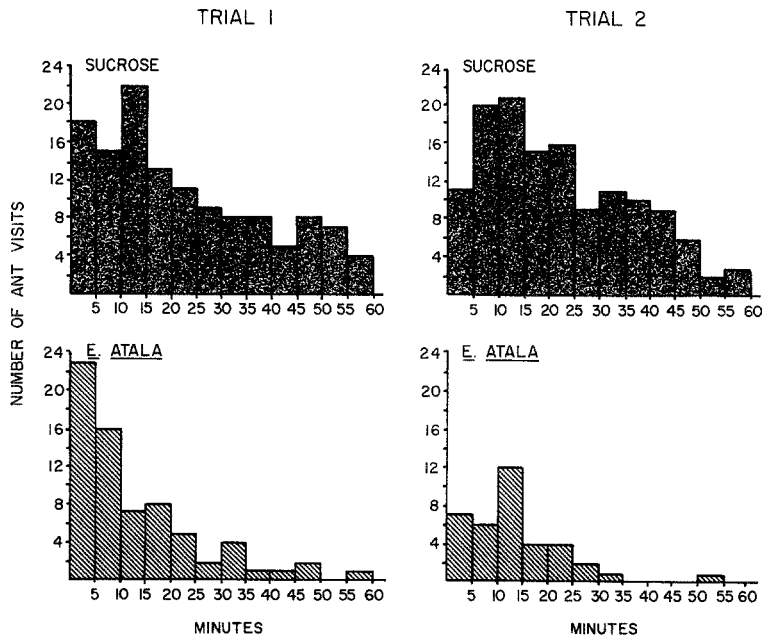


FIG. 5. The response of foraging *C. abdominalis floridanus* ants to a choice of drops of solutions of 25% sucrose and a dried, ground *E. atala* butterfly in 25% sucrose (8.5 mg/ml). The number of ants drinking from the drops was recorded every minute, but the data were combined for 5-min intervals for presentation.

that *E. atala* were unpalatable to these vertebrate predators (Bowers and Farley, unpublished data). Four adult jays were each offered three to six *E. atala* as part of a larger series of feeding trials. *Galleria mellonella* (Pyralidae) moths (six per bird) were used as palatable controls. All birds attacked and tasted at least one of the *E. atala* offered, but only one bird ate one. All birds ate all six

TABLE 2. NUMBER OF *C. abdominalis floridanus* ANTS VISITING TWO DROPS OF WATER, ONE CONTAINING DRIED GROUND *E. atala* BUTTERFLY AND THE OTHER CONTAINING DRIED, GROUND CRICKET IN WATER (THREE TRIALS, 1 HR EACH)

Trial	Cricket	<i>E. atala</i>	X ²	P
1	12	3	5.40	<0.025
2	19	3	11.64	<0.005
3	22	0	22.00	<0.005

G. mellonella offered. Chi-square analysis showed a significant difference in the number of *E. atala* versus *G. mellonella* that were eaten and not eaten ($\chi^2 = 36.00, P < 0.001$).

There are several mimics of *Eumaeus*. One of the most interesting is a newly described species of *Castnia* in the moth family Castiniidae (Miller, 1986). Members of the Castniidae are often brightly colored, day-flying moths, many species of which are Batesian mimics (Miller, 1986). This new species was collected in an area in Brazil where a species of *Eumaeus* was also flying. The mimic has the iridescent wings typical of *Eumaeus*, as well as the red abdomen and red spots on the ventral hindwing next to the abdomen which is typical of most *Eumaeus* (Miller, personal communication). Another mimic is *Hades noctula* (Riodinidae). This species has the red spot on the underside of the forewing next to the thorax instead of on the hindwing next to the abdomen, but otherwise is very similar.

Using TLC, we were unable to detect cycasin in the leaves of *X. floridana*, or in the larval frass of *E. atala*. This is apparently due to the relatively low concentration of cycasin in these samples, because cycasin was detected in both samples by GC: 0.1% dry weight cycasin in frass and 0.02% dry weight in the *X. floridana* leaf. Thus *E. atala* larvae are selectively sequestering cycasin from the host plant, where it occurs in relatively low amounts, and eliminating a small amount in the frass.

The feeding trials with *C. abdominalis floridanus* showed that the ants were deterred by the presence of cycasin in a sucrose solution that was normally attractive, as well as by the butterfly in water. However, as far as we could determine, the cycasin was not toxic to the ants (although there might be long-term effects that we could not distinguish). Ants are extremely numerous and important predators of insects in the subtropics and tropics (Wilson, 1971), where *Eumaeus* species are found. Our results suggest that ants may learn to avoid *Eumaeus*, and the gregarious habit and sedentary behavior of *Eumaeus* species may aid in this. For example, foraging ants might learn to avoid cycads containing *Eumaeus* larvae and/or pupae.

The behavior of the ants in response to *E. atala* and cycasin was very interesting. Only a small portion of the colony foraged in any trial, probably not more than 15 (distinguished by their distended abdomens). After drinking, the foragers returned to the nest and appeared to feed other ants and larvae. In both experiments using a sucrose solution, the ants initially foraged equally at the two solutions (control versus that with *E. atala* or cycasin), but after 10 or 15 min ceased visiting the solutions with *E. atala* or cycasin. This suggests that the cycasin and *E. atala* were not initially deterrent or that the attraction of the sucrose was so strong that the ants were not initially responding to the deterrents. Another explanation may be that the ants did not begin avoiding the experimental solutions until some physiological effect had occurred, such as

poisoning, or until something had been communicated within the colony as these solutions were transferred to other ants or larvae. In addition, our results show that the ants did not retain the information to avoid the cycasin solution over 24 hr because a second trial the following day elicited almost identical behavior of the ants—they again visited the control and cycasin solutions equally at first and after a short time ceased visiting the cycasin solution (Figure 2).

The aposematic coloration and behavior of *Eumaeus* species suggest that this advertisement is directed at visually hunting vertebrate predators; and, indeed, adult *E. atala* were unpalatable to gray jays (Bowers and Farley, unpublished data). Nonetheless, invertebrate predators (ants) also avoided *E. atala* adults after initial exposure to them. Thus *E. atala*, and probably other species of *Eumaeus* as well, provide the first demonstration of host-plant acquired chemical defense and unpalatability in the Lycaenidae.

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OLFACTORY ORIENTATION RESPONSES BY WALKING
FEMALE *Ips paraconfusus* BARK BEETLES
II. In an Anemotaxis Assay

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Abstract—Synthetic pheromone was released from a dispenser stretched across the width of a wind tunnel. Beetles in pheromone-free wind wandered in all directions and did not appear to orient to the wind. A dosage series showed that pheromone decreased the walking rate and deviations from the upwind direction, and it increased the turning rate. The tracks were composed of relatively straight or gently curving sections interspersed with more infrequent, larger course adjustments. Although pheromone clearly affected the average heading of beetles within a treatment, any given individual exposed to pheromone did not necessarily head directly upwind or maintain a fixed absolute angle with respect to the wind direction. The response appeared to be an inaccurate anemotaxis, rather than an anemomenotaxis.

Key Words—*Ips paraconfusus*, bark beetles, Coleoptera, Scolytidae, olfaction, orientation, anemotaxis, pheromones.

INTRODUCTION

The experiments described here were undertaken to develop an assay for quality discrimination by *Ips paraconfusus* among the three compounds that comprise its aggregation pheromone: *cis*-verbenol (cV), ipsenol (Ip), and ipsdienol (Id) (Silverstein et al., 1966; Wood et al., 1967). The key requirement for an assay was that the response be clearly modulated by the compounds of interest. Anemotaxis appeared to be a good candidate since the insects use the compounds

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for orientation in flight and since the upwind walking response has commonly been used to monitor attractancy (Wood and Bushing, 1963; Wood et al., 1966, 1967; Borden and Wood, 1966; Byers and Wood, 1981). Earlier assays generally employed a point source of odor, and the beetles might have been able to use differences in pheromone concentration between the pheromone plume and the surrounding uncontaminated air as one cue to guide themselves to the source (Kennedy, 1977b). Such chemotactic responses can be studied separately (Akers, 1985; Akers and Wood, 1989), and one of our goals was to have as many criteria as possible upon which to define odor discrimination. Therefore, an assay that permitted only anemotaxis and excluded chemotaxis was desirable. Chemotactic responses were avoided in the present assay by uniformly dispensing the pheromone across the width of a wind tunnel.

METHODS AND MATERIALS

Details concerning the compounds used as stimuli, the observation chamber, the recording procedures, the handling of the experimental animals, the experimental design and analysis of the experiments, and some of the analytical methods for orientation data have been described previously (Akers, 1985; Akers and Wood, 1989).

Compounds. Ipsenol, ipsdienol, and *cis*-verbenol were obtained from Booregard Industries, Ltd., Sarpsborg, Norway. Gas-liquid chromatography (GLC) showed the following purities: ipsenol 95%, ipsdienol 98%, and *cis*-verbenol >99%. None of the compounds had any contamination of the other compounds.

Observation and Recording Procedures. A beetle's movements were observed using a chamber, within which beetles oriented at random when no odor or wind was present. A beetle's movements were recorded by marking its position by hand at 1-sec intervals, with reference to an electronic metronome. If an animal stopped walking, recording of its position ceased until it began moving again. However, the beetles are highly thigmotactic, and most did not stop moving until they contacted the wall of the arena or the pheromone source, at which time tracking ceased. Tracks were later entered into a computer via a digitizing table.

Anemotaxis Assay. The design of the anemotaxis assay was based on the discussions of Kennedy (1977a,b, 1978) and was intended to eliminate possible chemotactic responses along the edge of an odor plume. Edges were avoided by dispensing the pheromone across the width of a wind tunnel.

The arena or working section of the wind tunnel was modified from the arena used in a previous chemotaxis assay (Akers, 1985; Akers and Wood, 1989). The top and bottom of the arena were each a pane of double-strength glass, 40.6 cm square. The top had a 1-cm hole in the center. Along the lengths

of two parallel edges of the plates ran aluminum rods, 1 cm square. This created an area $40.6 \times 38.6 \times 1$ cm, open on both ends. Heavy rubber bands along the open edges bound the arena together. One open end of the arena was slipped into the inlet manifold of the wind tunnel, completing the tunnel. The hole in the upper plate was sealed with a stopper except when access to the interior was needed.

A wind speed of about 0.9 m/sec was used in the assay because it is about midway in the range where *I. paraconfusus* is known to respond to pheromones in the field (Gara, 1963), and it is similar to wind speeds used in previous walking bioassays (0.76 m/sec in Wood and Bushing, 1963). Preliminary experiments showed no upwind responses to wind at 0.9 m/sec, in the absence of pheromone. Wind speed was measured at seven locations evenly spaced along the exit from the arena and was 0.94 ± 0.04 m/sec. The wind tunnel was isolated from the fan such that no vibration could be felt at the arena. Air temperature within the tunnel was $25 \pm 1^\circ\text{C}$.

The pheromone dispenser was a loop of six-strand, 100% cotton embroidery floss (J.P. Coats #1), 87 cm in circumference. This loop was attached at one point to a strong thread passing through the inlet manifold approximately 2 cm upwind of the arena. The stimulus could be presented at any moment by pulling the thread through the manifold. The dispenser then stretched across the entire width of the manifold approximately 0.4 cm above the floor of the arena. The stimulus was generally presented a few seconds after the beetles emerged into the wind stream.

Since a uniform stimulus across the arena was required, a uniform distribution of pheromone along the dispenser was necessary. The method described here was chosen because it gave a fairly uniform distribution, conserved pheromone compounds, and permitted an estimate of the dosage applied to the dispenser. The loop of floss was pressed into the bottom of a test tube. Enough test solution was applied to just thoroughly saturate the floss, with little or no excess. For the dispenser described, 0.7 ml was appropriate. The floss remained in the tube 3–4 min, then was stretched its full length. The solvent, pentane, evaporated in 1–2 min, and the dispenser was used immediately thereafter.

The distribution of the pheromone along the dispenser was determined by GLC. Linalool, 0.1 mg, was applied to each of four dispensers, using the method described above. Linalool was used to conserve pheromonal compounds and because its diffusion and evaporation properties are very similar to those of the pheromonal compounds (Table 1 in Akers and Wood, 1989). Five samples of floss, each 0.5 cm, were cut at 6-cm intervals along the length of each dispenser and were each extracted in 50 μl of pentane, to which an internal standard (decane) had been added, in order to check the measuring error. This would not include other sources of error such as variations in cutting the floss, or in the amount of pentane used to extract the samples. One microliter from

each sample was injected into the GLC. The coefficient of variation among the samples of a dispenser, as averaged over the four dispensers, was $22.3 \pm 6.8\%$ for the linalool and $16.7 \pm 4.2\%$ for the internal standard. These were not different at the 5% level in the *t* test. Should the observed means be the true population means, the variation from sample to sample along the floss would be about 15%.

The handling of each dispenser was relatively time-consuming. To test the effects of using a dispenser more than once, three dispensers were exposed in the wind tunnel for each of 0, 1, 3, and 5 min, and 0.1 mg of linalool was applied to each. After exposure, the dispensers were extracted individually in 2 ml of pentane for 20 min at room temperature, after which they were kept on Dry Ice. Two samples of $1 \mu\text{l}$ each were injected from each dispenser onto the GLC. About 60% of the pheromone evaporated in the first minute (Figure 1). Based on these results, a single dispenser was used only once in all behavioral experiments. Given the flow rate and dimensions of the arena, the average concentration in the air during the first minute of exposure was approx. 0.28 ng/ml.

A preliminary experiment showed that beetles did not exhibit any form of trail-following or otherwise interact with one another when more than one beetle was run within a single arena (Akers, 1985). In this experiment, the relationships between the tracks of two beetles that had run together within a single

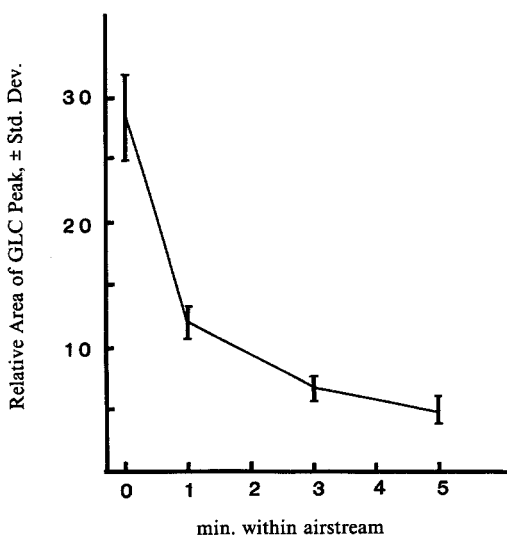


FIG. 1. The relative amounts of linalool remaining on the pheromone dispenser after differing durations of exposure to the wind stream of the wind tunnel used in the anemotaxis assay. The dosage on the dispenser was 0.1 mg.

arena were compared to the relationships between the tracks of a pair of beetles that had run in separate arenas. The average distance that the second beetle of a pair maintained with respect to the trail of the first beetle was measured, as were the walking and turning rates of the second beetle. There were no significant differences in these measurements between the beetles that had the opportunity to follow a trail and those that did not, in either the presence or absence of pheromone. Therefore, observations made on beetles tested within a single arena could still be considered as independent for statistical purposes, and testing more than one beetle in a single arena greatly reduced the time required per replicate. Ten beetles were usually run successively in each arena, with an interval of 2–3 min between them.

In order to minimize contamination between treatments, each treatment had its own arena. An arena was rinsed after each use in hot (approx. 70°C) water, flowing at a rate that provided an exchange of water approximately every 4 min. The rinse lasted about 40 min. The parts of an arena were then aired for three to five days before the next use.

Handling of Beetles. The beetles were collected from naturally infested ponderosa pine (Browne, 1972). Females were stored at 5°C in individual glass tubes, sealed with Teflon tape. They were removed from the cold 5–20 min before use, since their struggles within the storage tubes seemed to decrease their vigor after an hour or so. A beetle was brought out of the cold directly onto the observation platform of the observation chamber, where it recovered in 1–2 min, in the same light and temperature conditions under which it would be tested. A beetle was used only once.

When a beetle was to be released, the tape was removed from the storage tube and the beetle was slid gently but immediately into an L-shaped, glass release tube inserted through the hole in the arena. The direction of orientation of the outlet or the presence of wind did not have much effect on the consequent directions taken by the beetles (Figure 4 below). Nonetheless, the outlet of the tube was oriented approximately downwind, in an attempt to minimize random upwind movements. Since the pheromone components were at first thought to cause only attraction, the downwind release was intended to accentuate the difference between active and inactive treatments.

Experimental Design. A dosage series of a 1 : 1 : 1 blend of the three pheromonal compounds was used, as in earlier behavioral tests (Byers and Wood, 1981). Treatments were assigned in a randomized block design as a precaution against losing the supply of beetles during the experiment, but the effects of blocks were slight and are not reported. The treatments differed by powers of 10 and had a range of 10^{-7} –1 mg of each compound. The experimenter did not know the identity of the treatments, both during the experiment and during the manipulation of the data. Solutions were stored at –60°C or over Dry Ice until a few minutes before use.

Analysis of Orientation Data. The kinetic responses measured were the linear rate of motion and rate of turning. The estimate of the walking rate was the mean distance between each point on a beetle's track. Two related measures were used to summarize the turning rate. Both were based on the turn angle measured at each point on a track. The turn angle was defined as the angle between the direction from the previous to the current point on the track and the direction from the current to the next point. Zero degrees was defined as straight ahead from the current point on the track. For the "net turning rate," the "handedness" of a turn was taken into consideration, with left-hand turns defined as positive. In the "gross turning rate," the absolute value of each turn was used. The estimates of the rates were the mean turn angles over all the points on the track.

An insect's heading with respect to the wind was also measured at each point on its track. The heading was defined as the angle between the upwind direction and the direction from the present point to the next point on the track. The direction directly upwind was defined as 0°. Similar to the turning rate, a mean net heading and a mean gross heading were defined. In all, the data summaries included walking rate and its variation within a track, net turning rate, gross turning rate and its variation within a track, mean net heading, and mean gross heading and its variation within a track (Akers, 1985; Akers and Wood, 1989). The variations of net angle summaries were not examined because net angles are circular variables and the estimates of their variances have inherent statistical difficulties (Batschalet, 1981; Akers, 1985; Akers and Wood, 1989).

RESULTS

Increasing dosage decreased the walking rate (Figure 2A and Table 1), the mean gross heading (Figure 2G), and the magnitude of the mean net heading (Figure 2F); it increased the gross turning rate (Figure 2C) and its variability (Figure 2D). It also increased the variability of the mean gross heading (Figure 2H). Dosage did not affect the magnitude of the net turning rate or the variation of the walking rate. A beetle's track tended to have relatively linear or gently curving sections interspersed with sharper turns, even in the presence of pheromone (Figure 3). At the highest dosage, the tracks of some beetles became sinuous.

In a previous experiment on chemotaxis (Akers and Wood, 1989), a beetle had only a 2% chance of reaching the source by chance alone. Thus, when more than one or two beetles reached the source in a treatment, most of them were probably responding to the treatment. In the anemotaxis assay, no criterion could be found that satisfactorily distinguished responding from nonresponding beetles. First, in the absence of pheromone, the beetles apparently did not use

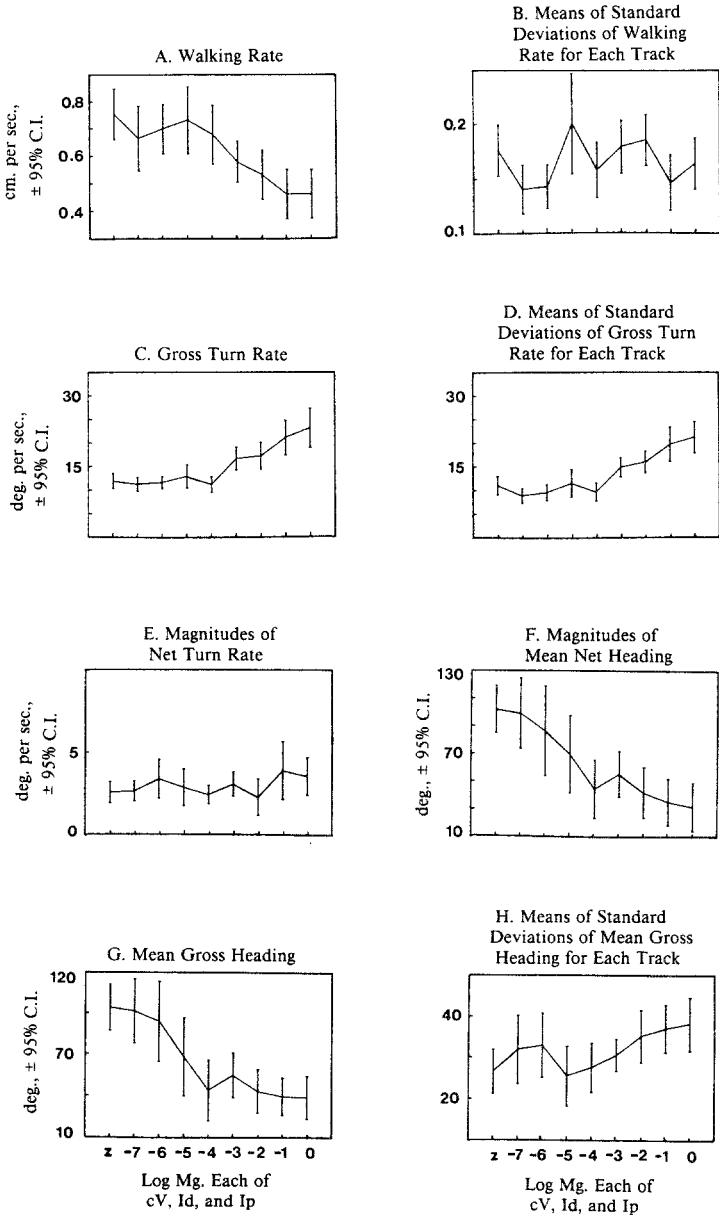


FIG. 2. The effects of dosage of a standard pheromone blend on measurements taken from the tracks of beetles in the anemotaxis assay. Ip = ipsenol, Id = ipsdienol, cV = *cis*-verbenol, z = zero dosage level. 95% C.I. = the 95% confidence interval of the mean.

TABLE 1. ANALYSES OF VARIANCE FOR EFFECTS OF PHEROMONE DOSAGES ON BEHAVIORAL MEASUREMENTS

Behavioral response	$F_{(obs)}$	P value ^a
Walking rate	5.44	<0.0001
Standard deviations of walking rates	1.48	0.168
Gross turning rate	12.20	<0.0001
Standard deviations of gross turning rates	12.99	<0.0001
Magnitudes of net turning rates	1.18	0.314
Mean gross headings	7.97	<0.0001
Standard deviations of mean gross headings	2.16	0.032
Magnitudes of net headings	7.31	<0.0001

^aThe P value is the probability of obtaining an F greater than the observed F , given that H_0 is true. H_0 : treatment means are not different from one another. N = a total of 218 beetles in nine treatments.

the wind for the purposes of orientation. They wandered in all directions after being released in a pheromone-free airstream, despite being released with a downwind orientation (Figure 4), similar to their random orientation in still air when no pheromone source was present (Akers, 1985; Akers and Wood, 1989). Further, even in high pheromone dosages, the angle any particular beetle maintained with respect to the wind was not necessarily directly upwind or even very nearly so (Figure 3).

Combined with the possibility that even an unstimulated beetle might head

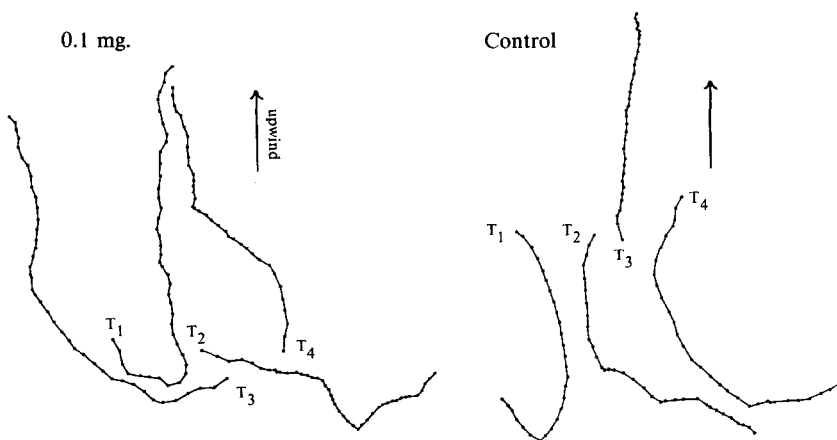


FIG. 3. Four sample tracks selected randomly from each of the control and 0.1-mg treatments. Points on the trails are 1 sec apart. T_x = the starting point of trail x .

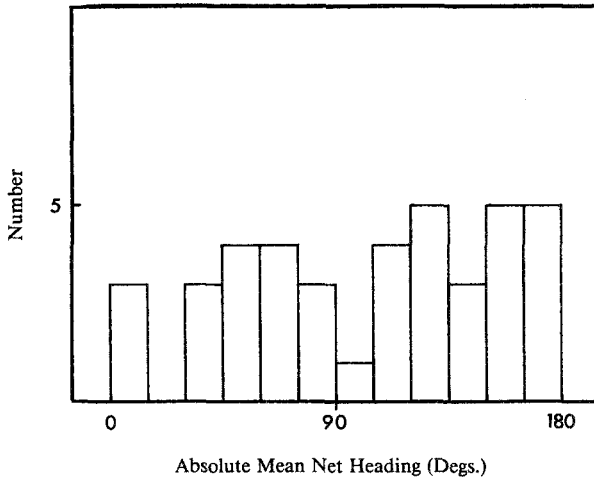


FIG. 4. A frequency histogram of the absolute mean net headings for the track of each of 40 beetles released into a clean wind stream in the anemotaxis assay. Beetles were originally released on a heading approximately downwind (180°).

upwind, the determination that any particular beetle had entered into odor-modulated anemotaxis became uncertain. For example, no criterion independent of heading, such as walking or turning rate, separated the beetles into two groups within which all or most of the beetles either did or did not orient strongly upwind. When criteria were developed based on heading, walking and turning rates were significantly different between the groups that had been provisionally identified as responding or not responding, but there were also significant differences among dosages within each group. Graphs of the data also showed similar trends within each group. For instance, gross turning rate tended to increase with dosage and walking rate decreased, even among the treatments in the group that had provisionally been classified as not responding to the pheromone. Furthermore, heading apparently decreased with dosage in this group. Results of this sort led us to believe that, while we could demonstrate strong effects on the responses of the population, we could not safely identify individual beetles as responding or not responding to the pheromone.

DISCUSSION

The results of this assay are very similar to those obtained in the chemotaxis assay (Akers and Wood, 1989), except that turning rate (Figure 2C) and its variability (Figure 2D) and mean gross heading (Figure 2G) and its variability (Figure 2H) were lower in the anemotaxis assay. These results reflect

the impression that the tracks were more linear in the anemotaxis assay (Figure 3) than in the taxis assay (Figure 7 in Akers and Wood, 1989). The tracks often seemed to be composed of relatively straight or very gently curving sections, interspersed with a few relatively large course adjustments. This is similar to observations on *Bombyx mori* and *Grapholita molesta* (Kramer, 1975; Willis and Baker, 1987). However, the beetles did not maintain fixed absolute headings with respect to the wind, while *B. mori* did so both in clean wind and in wind uniformly contaminated with pheromone. Neither did the beetles consistently head as directly upwind as the tracks of *G. molesta* illustrated in Willis and Baker (1987). However, since the latter work employed a point source of pheromone, perhaps a loss of the pheromone plume corrected any tendency of the moths to wander from a path straight upwind. Furthermore, the beetles were released facing downwind only about 22 cm below the pheromone source, whereas *G. molesta* was released about a meter from the pheromone source. Although the beetles usually took at least 30 sec to cover the distance to the source, perhaps their performance would have improved with more time.

When beetles were exposed to pheromone, the shape of their tracks often approximated those of control beetles, but their directional vectors had shifted upwind (Figure 3) (Bell and Tobin, 1982). In some cases, the beetles exposed to pheromone seemed to have a fairly high turning rate early in the track, while the later portions became more linear as the track approached the upwind direction (e.g., track T₁ in Figure 3). However, scattergrams and exploratory regressions of turning rate against time on the track or against percent of the total period of the track did not in general support the notion that turning rate slowed as the track was run. It seemed to be more a function of the individual beetle.

Wind was the only orienting cue available to the beetles in these experiments because the pheromone was released uniformly across the width of the windstream. The lower headings with respect to wind direction in the presence of pheromone (Figure 2F,G), and the linearity of the tracks (Figure 3) relative to the chemotaxis assay (Figure 7 in Akers and Wood, 1989), lead to the conclusion that the response is an odor-modulated orientation with respect to the wind direction (anemotaxis). The broad curves and course changes in the tracks indicate the beetles were not executing a compass orientation with respect to the wind (anemomenotaxis), as *B. mori* had.

The threshold dosage for the response appeared to be about 10^{-6} – 10^{-5} mg, while in the chemotaxis assay the threshold was no higher than 10^{-4} mg (Akers and Wood, 1989). A dose of 0.1 mg linalool produced an average pheromone concentration of 0.28 ng/ml in the air of the arena in the anemotaxis assay (see Methods and Materials), while a dose of 1.0 mg produced a concentration of 5.8 ng/ml at the release point of the beetles in the chemotaxis assay (Table 1 in Akers and Wood, 1989). Accordingly, the concentration in the air for a given dose of pheromone may have been similar in the two assays, making

these thresholds remarkably close. The threshold is lower in the anemotaxis assay, as might be expected since the odor does not have to provide directional cues. However, the low threshold in the taxis assay once again attests to the relative strength of that response in the beetles.

The results from the present anemotaxis assay are somewhat different from the results of earlier studies (Wood and Bushing, 1963; Borden and Wood, 1966; Byers and Wood, 1981) in which a point source of pheromone was released into a broad airstream. In those studies, the behavior of the beetles was not quantitatively described beyond noting whether they arrived at the source. However, descriptions and examples of the tracks indicate that they were fairly sinuous, so much so that the response was characterized as a chemoklinotaxis (Wood and Bushing, 1963; Borden and Wood, 1966). Although they were sinuous, the tracks did not appear to wander very far from a line connecting the release point of the beetles and the source of pheromone directly upwind of it, much like the tracks of *G. molesta* walking to a point source of pheromone (Willis and Baker, 1987). That is, the beetles seemed on average to head more directly upwind than in the present study. One potential explanation for the beetles' sinuous paths in the earlier studies might be that they execute an endogenously generated series of turns upon encountering pheromone-laden air. Some moths apparently initiate endogenously generated zigzagging when they encounter pheromone (Kennedy et al., 1980), although the control of turning by flying moths appears once again to be a matter of some controversy (Preiss and Kramer, 1986; David and Kennedy, 1987; Willis and Baker, 1987). In the present study the stimulus was presented a few seconds after the beetles emerged into the arena, and there was no sign of zigzagging or especially rapid turning early in the tracks (Figure 3). Apparently, then, the sinuous upwind responses observed in earlier studies were not purely anemotactic and were not zigzagging analogous to that of moths. Instead, some form of taxis may have been occurring along the border between clean and pheromone-laden air downwind from the point source. However, the behavior of the beetles upon emerging from pheromone-laden air needs to be studied before a cogent theory can be constructed as to the mechanism of orientation to a point source in wind. Perhaps a loss of pheromone stimulation initiated the sinuous movements seen in the earlier studies. Alternatively, some form of counterturning initiated by the loss of pheromone could be involved (Akers, 1985, 1989).

While this work was done with walking insects, the results have similarities to those obtained with flying beetles. Studies of anemotaxis with beetles flying in wind tunnels are scarce. One reason appears to be that beetles will not zigzag nicely within a wind tunnel, as will moths. Instead, they tend to take off each in their own particular direction and then fly in a fairly linear manner until they collide with the walls of the chamber. The addition of pheromone shifts the general direction of take-off towards the upwind direction, but the beetles

still do not zigzag and the direction of take-off seems to depend strongly on the individual (Choudhury and Kennedy, 1980), which is similar to our results.

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LOCATION OF VOLATILE ODOR SOURCES BY GHOST CRAB *Ocypode quadrata* (Fabricius)

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Abstract—The ghost crab, *Ocypode quadrata*, was tested in the field for its ability to locate sources of volatile cues. The pure compound skatole, 3-methylindole, was a potent attractant. Crabs also located sources of complex odors such as dead fish, *Lutjanus campechanus*, dead mole crabs, *Emerita talpoida*; and peeled bananas. Ghost crabs possess concealed and reduced antennules that may not be the primary olfactory organs. Chemosensory hairs borne on the dactyls may be the primary detection system.

Key Words—*Ocypode quadrata*, ghost crab, olfaction, chemoreception, crustacea.

INTRODUCTION

Study of signaling functions of volatile compounds in complex mixtures for crustaceans is confounded in water by the presence of water-soluble nonvolatile cues. The study of responses of terrestrial crustaceans to airborne cues enables isolation of volatile from nonvolatile compounds in complex mixtures. Using this technique, Rittschof and Sutherland (1986) demonstrated that land hermit crabs *Coenobita rugosus* can use volatile chemicals to locate fruit, carrion, and feces.

We extended that study to another crustacean, namely the ecologically similar yet phylogenetically distant, semiterrestrial ghost crab, *Ocypode quadrata*. Ghost crabs live in burrows scattered from the intertidal to hundreds of meters inland. They emerge from their burrows shortly after dark to scavenge, predate, and reproduce. Ghost crabs eat live prey, including mole crabs (*Emerita talpoida*), coquina clams (*Donax variabilis*), and conspecifics (Wolcott,

1978). We have seen them scavenge horse manure, horseshoe crabs, sea gulls, cooked shrimp, peanuts, potato chips, candy wrappers, and beach wrack. Bearing in mind their adaptation to the terrestrial environment, we asked the question: can these crabs also use volatile cues to locate food?

METHODS AND MATERIALS

Study Area. Two 120 × 20-m areas of beach on Bird Shoal, immediately due East of the Beaufort Inlet, North Carolina, were field sites chosen because searches showed these areas were more densely populated with ghost crabs than were the adjacent stretches of shore. One area included approximately 60 m of the western shoreline, before the southernmost point, and 60 m of the southern shoreline. Dredge spoil dunes bordered this stretch of beach. Most of the dunes had dune grasses (*Spartina patens* and *Uniola paniculata*) growing on them. Burrows of crabs were located from the high intertidal to well within the dunes. The other site was 300 m east of the first, on the southern shore. This site had a few dunes to the west but for the most part was a flat sand isthmus between a low energy ocean beach and a tidal flat. Crab burrows were scattered throughout the isthmus area above the high tide line.

Odor Sources. Six odor sources, two each from the categories of animal, plant, and feces, were tested in the field. Five sources [red snapper (*Lutjanus campechanus*) and mole crabs; peanut butter and bananas; and horse manure] generated complex odors and were chosen because they mimic naturally occurring ghost crab food. Bananas, fish, and horse manure have previously been shown to be attractive to the land hermit crab (Rittschof and Sutherland, 1986). The pure compound, skatole, 3-methylindole, is a known component of feces (Stecher, 1960).

Field Assay. Rittschof and Sutherland's (1986) field assay was modified to test the ability of ghost crabs to locate substances using only olfaction. Bait was concealed in a cylindrical hole 10 cm in diameter × 15 cm deep. Each hole was covered with 1 cm mesh galvanized hardware cloth and marked with a surveyor's flag. Because of its design, the hole eliminates the possibility of a crab finding the bait by sight, taste, or touch. Thus, a crab locates the hole by chance or through the use of olfaction. Unbaited holes were used as controls (C), which were placed on either side of experimentals (E) to make an E, C, E, C, transect. Each assay had equal numbers of control and experimental holes. A goodness-of-fit test was used to compare the frequency of responses to each baited trap series. Test series were conducted until the minimum number of responses in any category was five, in order to satisfy the assumptions of the statistical analysis. The null hypothesis that responses were independent of the

bait was tested using a G statistic with 1 df after correction for continuity (Sokal and Rohlf, 1981).

Baited holes contained one fish head (6–10 hr old), which weighed approximately 100 g; several (three to four) mole crabs (total weight 25 g); or one to two pieces of horse manure (50 g). One filter pad (4.25 cm in diameter), soaked in a saturated skatole–seawater solution, was used per hole. One quarter of a peeled banana (40 g) was used and one tablespoon of Superman brand peanut butter was used (20 g) per hole.

Usually, 40 holes were placed in a horizontal transect every 3 m, just below the high water mark. Data were pooled from two to six nights using the same test substances. Holes were dug in an interval 30 min before sunset and baited 15 min before the beginning of the assay. Assays were generally run for 90 min and began just after dark. The line of holes was checked every 15 min using a 6-V dive light (bulb: GE H7550 halogen; changeable Technacell 6-V battery EP640). Any crab found touching the wire mesh cover was collected and held until the end of the assay to prevent counting any animal twice. To rate the attractiveness of the substances, tests were normalized with respect to time and surface area for odor release. First, all control responses were normalized with respect to the lowest value. Each substance's experimental response was divided by its normalized control value giving an attractiveness adjusted for crab activity. Then, the time-adjusted attractiveness figure was divided by the approximate surface area of the odorant.

Observations. Crab activities were observed over three summers (May to October 1985–1987). Initially, assays were conducted on a schedule, but after two seasons, field assays were conducted immediately after dark only on nights when, in our experience, crabs were highly active. Crab activity was assessed visually and correlated with wind speed, temperature, time of the tide, and phase of the moon. Crabs outside the boundaries of the study areas were observed during other times, primarily to determine what they were eating. Because observations of ovigerous females were extremely rare (T. Wolcott, personal communication), these were actively searched for.

To study the response of the crabs to volatile cues, crabs were observed approaching a hidden odor source. One $30 \times 30 \times 15$ -cm hole was dug in the middle of site 1, just below the high water mark, 15 min before dark. Red snapper (1 kg), known from assays to be attractive, was placed in this hole. A dim red light (600 nm) was fastened to the top of a wooden pole buried in the sand 30 cm from the hole, so that the light was 1 m above the sand. Ghost crabs were not predicted to see this wavelength of light (Cronin and Forward, 1988). The illuminated area was about 3.0 m in diameter. A yarn tell-tale was tied to the top of the pole to gauge wind direction. The observer sat 2 m upshore of the hole, facing the water. In this position the prevailing wind direction was

from the observer's right to left. For 1 hr, beginning 30 min after sunset, the approach of crabs to the hole was observed. Direction, pattern of movement, body posture, and general behavior of the crabs were noted.

RESULTS

General Observations. During May through September, ghost crabs leave their burrows shortly after dark. They remain near the burrow mouth briefly and then either move to the water to forage along the tide lines or to actively pursue prey (*Donax* and *Emerita*) at the water's edge. Most crabs caught feeding in the surf zone were eating *E. talpoida*. Copulations were observed approximately 10% of the nights. Cannibalism was observed approximately 5% of the nights. At the new and full moon, hatched eggs and inactive larvae were found on females before they entered the water. Larval release was after dark within 40 min of high tide. When temperatures were below 20°C, crabs were inactive. Activity was reduced on nights with wind greater than 17 km/hr.

The paths of nine crabs were recorded during the 90-min interval in which the single pit baited with 1 kg of red snapper was observed. Each crab was followed closely when it approached within 3 m of the pit. Seven of the crabs approached from downwind of the bait. These crabs either moved directly to the pit or moved upwind, paused, moved 10–30 cm laterally, and then moved directly to the pit. All crabs had an elevated body posture, accomplished by extension of the walking legs to approximately 60% of maximum. All eventually stood directly on top of the odor source and attempted to feed. At this point crabs were captured. Two crabs approached the pit from the upwind direction, came within 60 cm of the pit and walked by. Neither crab showed the elevated body posture, and both crabs passed by without stopping.

Field Assay Responses. As previously mentioned, the data have been divided into the categories of carrion, plants, and feces. The responses of crabs to pitfalls baited with animal flesh (Figure 1A) were 3.6 times greater than the controls for mole crabs ($G = 7.8$, $df = 1$, $P < 0.01$) and 3.0 times the response to controls for red snapper ($G = 8.8$, $df = 1$, $P < 0.01$). The experimental to control ratio for responses to plant material (Figure 1B) was slightly lower than that for carrion. Crabs responded significantly to banana ($G = 9.0$, $df = 1$, $P < 0.01$), but not to peanut butter ($G = 3.7$, NS). In the feces category (Figure 1C) the response to skatole was 3.0 times greater than controls ($G = 9.4$, $df = 1$, $P < 0.01$), whereas the response to horse manure did not differ from that of the controls ($G = 1.7$, $df = 1$, NS).

Relative Attractiveness to Volatile Stimuli. Relative attractiveness data are

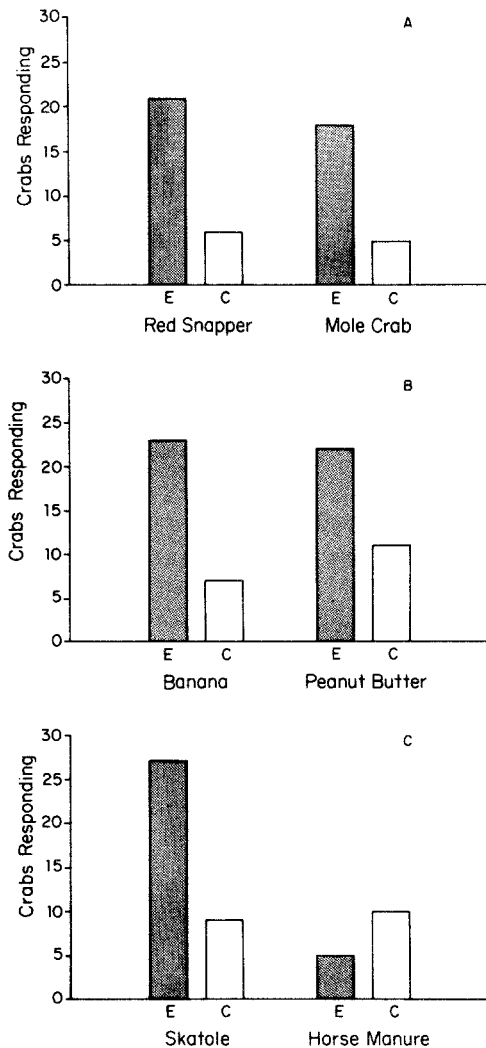


FIG. 1. (A) Field responses of ghost crabs to the complex odors of two animal remains: red snapper (*Lutjanus campechanus*) and mole crabs (*Emerita talpoida*). E is the response to flesh; C is the response to control. (B) Field responses of ghost crabs to the complex odors of two plants: banana and peanut butter. E represents the response to plant odors; C is the response to controls. (C) Field responses of ghost crabs to the pure compound skatole, 3-methylindole, and the complex odor of feces represented by horse manure. E signifies the response to odors; C is the response to controls.

TABLE 1. ADJUSTED ATTRACTIVENESS OF TEST SUBSTANCES TO GHOST CRAB

Substance	Adjusted attractiveness	Surface area (cm ²)	Relative attractiveness (adjusted attractiveness/surface area)
Skatole	10.0	14.2	0.7
Mole crab	13.0	50.3	0.3
Banana	11.4	201.1	0.06
Snapper	12.5	706.9	0.02
Peanut butter	5.0	201.1	0.02
Horse manure	-2.5	201.1	-0.01

summarized in Table 1. Skatole and mole crabs were the most attractive. Bananas, snapper, and peanut butter were an order of magnitude less attractive. Horse manure was not attractive.

DISCUSSION

Detection of volatile odors would enhance the ability of terrestrial scavenging crabs to function. An omnivorous scavenger would be expected to respond to odors that signal potential food sources. Substances from three categories of odors: animal, plant, and feces, were attractive to ghost crabs. Skatole, mole crabs, banana, and red snapper were significantly attractive. Peanut butter and horse manure were not. Use of volatile compounds as food cues is thus a certainty. The response of ghost crabs to odor cues or pheromones for other purposes remains to be demonstrated.

Scavengers and predators should respond to a wide range of odor cues and could be responding for two reasons: a crab may investigate an unusual odor or cues that are characteristic of food sources (Hazlett, 1971; Robertson et al., 1980, 1981). Decarboxylated amino acids such as skatole, putresceine, and cadaverine are primary by-products of bacterial decomposition (Trott and Robertson, 1984) and are characteristic of high nitrogen sources such as carrion and feces. Decarboxylated amino acids cue cheliped flexion in ghost crabs (Trott and Robertson, 1984) and, as in the case of skatole, may also attract crabs from a distance.

The field assay tested whether or not a crab investigated an odor source. The field assay is a measure of a directed response evoked after odor detection. The extent to which a crab investigates an odor may depend upon the source's

desirability as a food item. Changes in sensitivity to common odors dependent upon dietary intake would diversify diet. Rittschof et al. (1985) demonstrated that if fed a certain food for several days, *C. rugosus* did not respond behaviorally to the odor of that food. It is possible that ghost crabs can smell horse manure but do not respond to the odor. Horses live in the area of the study, and manure is not a limiting factor. In manure-limited situations, ghost crabs may also respond to manure as do hermit crabs (Rittschof and Sutherland, 1986).

Ghost crabs responded to skatole and not to horse manure. Manure is a complex odor that includes skatole (Stecher, 1960). This inconsistency can be explained by the dietary intake argument described above (ghost crabs have been observed dining on manure). If sensitivity to odorants is decreased with exposure, as observed with aquatic snails (Rittschof et al., 1984), then extremely high levels of skatole (saturated solutions) might overcome the loss in sensitivity due to consumption. Another possibility is that other components in manure interfere with the response.

Wolcott (1978) reported that on high-energy beaches 91% of the ghost crab's dietary intake comes from predation. He emphasized that ghost crabs are also facultative scavengers. On lower-energy ocean beaches such as our study site, *E. talpoida* is less prevalent and *D. variabilis* is scarce. *E. talpoida* and *D. variabilis* constitute 87% of prey species in Wolcott's study. At our site, crabs caught feeding in the surf zone were eating *E. talpoida*. Scavenging appeared to be a prominent strategy in our study area. Although ghost crab eyesight is impressive (Daumer et al., 1963), observation of ghost crabs shows that they consistently obtain information through other senses, such as touch and olfaction. The use of the other senses is most apparent in situations where vision has little input. Vision by compound eyes is limited at long distances and obviated at short distances by the location of the target in relation to the eyes (Cronin, 1986).

Wolcott (1978) reported that crabs moved to the water immediately upon emerging from their burrows. We observed animals lingering near the burrow mouth upon emerging. Trott (1987) stated that *O. gaudichaudii* can maximize net energy intake by correlating foraging intensity with food density. This same strategy appears at our site, but the crabs are using olfaction as well as their contact chemoreception. We hypothesize that upon emerging from their burrows, the crabs first "test the air" for a potential food source. If they smell something on the beach or in the drift-line, they investigate. If not, they go on to the surf line and prey upon *E. talpoida* and *D. variabilis*. Crab approach patterns in response to odors are similar to those of moths flying to an odor source (David et al., 1982) and tube-nosed birds to wick-emitting volatile odors (Hutchison and Wenzel, 1980). These patterns suggest an integrated upwind approach to an odor source.

Banana and skatole, two odors that we have shown to be attractive to ghost crabs, are also attractive to *C. rugosus* (Rittschof and Sutherland, 1986). Hermit crabs and ghost crabs evolved from different aquatic ancestors. The responses observed could be a result of convergent evolution, or detection of volatile cues may have been a capability of an aquatic ancestor.

Observations suggest that land hermit crabs detect volatile cues with their large foliose antennules (Rittschof and Sutherland, 1986). Ghost crab antennules are greatly reduced and concealed behind the rostral plate in an aqueous medium. We find it unlikely that the antennules are solely responsible for orientation to volatile cues and hypothesize instead that hairs on the dactyls function in odor detection. Dactyl hairs have already been shown to act as contact chemoreceptors (Trott and Robertson, 1984). The leg hairs of the American lobster (*Homarus americanus*) have also been shown to have such functions (Derby and Atema, 1982). When orienting to an odor source, crabs were observed to maintain a higher body posture than at other times. Perhaps this posture could give better air flow to the dactyl hairs.

If crustacean food search can be mediated by volatile cues, then volatile compounds may also mediate other major biological functions such as mating. We pose the hypothesis that ghost crabs use a pheromone communication system similar to that of terrestrial insects. The use of volatile compounds as pheromones by crustaceans is an area that has not been investigated. Future studies will use receptive male and female crabs as "bait" in the holes to test this hypothesis.

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COLONIZATION OF SOUTHERN BEECH BY *Platypus caviceps* (COLEOPTERA: PLATYPODIDAE)

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Abstract—The role of host- and beetle-produced odors in the colonization of southern beech (*Nothofagus* spp.) by the pinhole borer *Platypus caviceps* Broun was investigated. Host-selecting males attacked the crown zone of a recently felled tree. Beetle emergence and dispersal were influenced by temperature, and sparse colonization continued over the 30 days of the study. Field tests using naturally baited traps indicated that male colonization of southern beech can be accounted for by attraction to host odors alone and that subsequent female response is to a male-released sex pheromone acting alone or in combination with host odors.

Key Words—Host colonization, sex pheromone, ambrosia beetle, *Platypus caviceps*, Coleoptera, Platypodidae, *Nothofagus*, southern beech.

INTRODUCTION

The pinhole borers *Platypus caviceps* Broun, *P. apicalis* White, and *P. gracilis* Broun attack healthy or stressed living and recently felled southern beech (*Nothofagus* spp.) in New Zealand. *P. apicalis* and *P. gracilis* attack a wide range of hosts whereas *P. caviceps* attack is almost solely restricted to southern beech. As possible vectors of a pathogenic fungus (*Sporothrix* sp.) *Platypus* beetles are a major cause of death and hidden decay among beech trees. Because of this relationship, *Platypus* beetles influence the economics of management of New Zealand beech forests. As the world's hardwood resource dwindles and attempts to manage natural and planted stands increase, understanding the behavior of *Platypus* beetles could be vital.

Primary attraction in the genus *Platypus* has been associated with host

odors, particularly ethanol and/or monoterpenes (Samaniego and Gara, 1970; Elliot *et al.*, 1983; Shore and McLean, 1983; Fatzinger, 1985). After initial attack by the host-selecting sex (*Platypus* males), subsequent buildup of attack has been associated with male-released aggregation pheromones (Madrid *et al.*, 1972; Renwick *et al.*, 1977; Milligan, 1982; Milligan *et al.*, 1988; Milligan and Ytsma, 1988).

Of the New Zealand species, *P. apicalis* and *P. gracilis* exhibit mass attack behavior mediated by aggregation pheromones (Milligan, 1982; Milligan *et al.*, 1988; Milligan and Ytsma, 1988; Ytsma, 1988). *P. caviceps*, however, attacks only sparsely, suggesting that aggregation pheromones may not be used during host colonization. This paper describes the role of host- and beetle-released odors in *P. caviceps* attack on beech and gives information on the behavioral pattern of attack.

METHODS AND MATERIALS

Two field studies were carried out during January–March 1987 in beech forest dominated by *Nothofagus solandri* (Hook.f.) Oerst. in Ashley State Forest, Canterbury, New Zealand, where a ground survey indicated a low- to medium-level population of *P. caviceps*. These studies were designed to (a) observe the colonization behavior on a felled tree, and (b) investigate the role of host- and beetle-released odors as attractants.

Colonization of Felled Tree. The colonization of a previously unattacked healthy, felled beech tree (dbh = 0.41 m, ht = 10.2 m) was observed. Fresh attacks were counted and marked daily between 1400 and 1600 hr for the first 30 days after the tree was felled. Two multiple-funnel traps, similar to those designed by Lindgren (1983) but using only six funnels, were placed either side of the base (0–3 m), center (3–6 m), and crown (6–10 m) of the felled tree to sample incoming beetles. Beetles were removed from the traps daily, counted, and sorted by sex.

Field Trapping. Logs infested with *P. caviceps* were obtained from west coast beech forest and transported to our Rangiora field station where they were stored under black polythene. Approximately equal numbers of adult males and females emerged randomly from November to May (Ytsma, unpublished data). Beetles were collected from these source logs (Milligan, 1982), and the males were induced to attack billets of beech (Ytsma, 1986). Males were allowed two days to establish tunnels, as preliminary tests indicated that before this time few males in tunnels were attractive to female beetles (i.e., they had not begun pheromone release). Infested billets containing about 25 male attacks and similar uninfested billets cut from the same logs with clusters of 25 holes drilled in each were used as baits for field trapping.

At each of three sites (50 m apart) multiple funnel traps that intercepted beetles 25–100 cm above ground level were suspended over an infested billet, an uninfested billet, and on an unbaited control, each set grouped to form a triangle with 3-m sides. The billets were wrapped in gauze to prevent fresh beetle access. Catches were removed daily, counted, and sorted by sex. If, after four consecutive days, no more beetles were trapped at a site, fresh billets were used and trap positions rerandomized. Since the data from each site were not significantly different, they were pooled for analysis. Means were compared for significant differences using the paired *t* test and Student's *t* test (Snedecor and Cochran, 1980).

RESULTS

Colonization of Felled Tree. Within two days of felling, the foliage began to wilt and the first two attacks were detected. A total of 56 sparse attacks occurred during the 30-day postfelling observation period, with 49 (70%) located in the crown zone. The base of large branches was often the site of attack in the crown. No "spillover" of attack was found in standing trees near the felled tree.

Females were usually found occupying the tunnels of males within three to eight days after attack. Apart from two males that survived in their tunnels for about 15 days, no live males were found in tunnels without a female. Of the marked tunnels, 11 (20%) were empty or had only a female occupant. This may be the result of predation by insectivorous birds.

A total of eight male and 24 female *P. caviceps* were intercepted by the traps placed adjacent to the felled tree for a period of 30 days. Most beetles (five males and 17 females) were caught in the crown zone, with only two males and eight females, and one male and one female caught in the center and base zones, respectively. The predominance of females caught in traps may be due in part to the presence of a male-released sex pheromone attracting only females and a tendency for males (the pioneer sex) to disperse to new hosts.

Most males observed flew and located the felled tree between 1500 and 2000 hr, although seven attacked between 1000 and 1200 hr. Flight activity of *P. caviceps* observed in the field coincided with the emergence of beetles from source logs used to infest the billets. The morning activity observed for some beetles could be accounted for if beetles did not always fly directly to colonize the felled tree but spent some time in the forest duff. These beetles would resume activity earlier the following day than freshly emerged beetles because they were not buffered from temperature changes by being deep within the tree, but were able to fly immediately when ambient conditions were suitable.

During a four-day period of comparatively warm weather (26–31°C), there

TABLE 1. MEAN NUMBERS OF *Platypus caviceps* CAUGHT IN FUNNEL TRAPS SUSPENDED ABOVE UNINFESTED OR MALE-INFESTED BEECH BILLETS (ASHLEY FOREST, JANUARY-MARCH 1986)

Treatment	Males (mean \pm SE) ^a	Females (mean \pm SE) ^a	Replicates ^c
Uninfested billet	2.8 \pm 0.66	1.3 \pm 0.27 ^{*b}	15
Male-infested billet	1.8 \pm 0.44 ^a	51.6 \pm 7.04 ^{c***}	15

^aMeans within columns are significantly different at $P < 0.05$ (a) or $P < 0.001$ (c) by paired t test.

^bResponses of sexes significantly different at $P < 0.05$. (*) or $P < 0.001$ (***) by Student's t test.

^cReplicate = catch per trap per 5- to 10-day interval.

were 23 attacks, representing 41% of the total attacks, and two males and 10 females representing 38% of the total beetles were intercepted in the funnel traps. No fresh attacks were observed, or beetles trapped, while temperatures were $< 12^{\circ}\text{C}$. This suggests that emergence may be clumped, depending to some extent on ambient temperatures.

Field Trapping. More males than females were attracted to uninfested billets but females dominated the catch in traps above male-infested billets ($P < 0.001$) (Table 1). In addition, significantly more males were caught in traps above uninfested billets than in those above male-infested billets, and significantly fewer females were caught in traps above uninfested billets than in those above male-infested ones. Neither sex responded significantly to unbaited (blank) traps as only one male and one female were caught during 15 replications.

Infested billets remained attractive to beetles for about seven to 10 days, with 26 males and 663 females (86% of the total catch) being caught during their first six days in the field.

DISCUSSION

The strong preference of *P. caviceps* for the crown zone of the felled tree may be due to the release of volatiles from this most rapidly drying (water stressed) zone. In beech forests, *P. caviceps* often survives in drier material than *P. apicalis* or *P. gracilis* (Ytsma, unpublished data). The gradual buildup of isolated attacks over time suggests that the males responded primarily to tree condition. Previous observations (Ytsma, unpublished data) have indicated that *P. caviceps* males prefer to attack trees with the highest growth rates in a stand,

possibly because more fissuring of the bark releases more volatiles. Roberts (1968, 1969) found a similar preference in other platypodids: *P. hintzi* Shauf, *Doliopygus serratus* Strohm, and *Trachyostus ghanaensis* Schedl, as did Milligan (1979) for *P. apicalis* and *P. gracilis*. This suggests that high local concentrations of phloem volatiles may be important in the siting of *Platypus* attack (Ytsma, 1986). The pattern of attack seemed to be related, at least to some extent, to ambient temperatures, as has been found for many other bark and ambrosia beetles.

Like the other New Zealand *Platypus* species, males of *P. caviceps* were attracted by host odors and were the tunnel-initiating sex. However, unlike the other two species (Milligan, 1982; Milligan et al., 1988; Milligan and Ytsma, 1988; Ytsma, unpublished data), *P. caviceps* males in tunnels attracted predominantly females, and male response could be accounted for by attraction to host odors alone. This is clear evidence that *P. caviceps* males release a sex pheromone rather than an aggregation pheromone. This has not been previously recorded for Platypodidae.

Although attack was sparse and scattered over time, nearly all the live males in tunnels had female partners. This suggests that the male-released pheromone is efficient in attracting females and does not require the presence of large numbers of males to be effective. Roberts (1968) also found that it was unusual among the sparse attacks of *T. ghanaensis* to find a male alone in its tunnel. The pattern of response to host odors and male-released sex pheromone described for *P. caviceps* may therefore be characteristic of other ambrosia beetles that do not aggregate during colonization of the host.

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STRUCTURES AND POSSIBLE FUNCTIONS OF
EPOXYPUKALIDE AND PUKALIDE
Diterpenes Associated with Eggs of Sinularian Soft Corals
(Cnidaria, Anthozoa, Octocorallia,
Alcyonacea, Alcyoniidae)

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Abstract—Chemical analysis of the eggs spawned by five *Sinularia* soft corals (Octocorallia, Alcyoniidae) collected in the central region of the Great Barrier Reef, Australia, revealed the presence of the known cembranoid diterpenes epoxy pukalide and/or pukalide in all cases. Examination of the colonies from which the eggs were released one month before spawning and also some time after spawning failed to detect either diterpene in the coral tissue. The three dimensional structures of pukalide and epoxy pukalide, as determined by the single-crystal X-ray technique, are reported for the first time. The wide distribution of pukalide and epoxy pukalide and of related compounds within the octocorallia is discussed.

Key Words—*Sinularia* spp., spawning, soft coral, pukalide, epoxy pukalide, structure determination, diterpene, octocorals.

INTRODUCTION

Soft corals (Cnidaria, Anthozoa, Octocorallia) have proven to be an extremely rich source of terpenoid natural products (Tursch et al., 1978; Faulkner, 1984,

1986, 1987). A number of studies have implicated secondary metabolites in the chemical defence of soft corals, either as toxins (Bakus, 1981; Tursch, 1982; Coll et al., 1982b; Coll and Sammarco, 1983) or as feeding deterrents (Tursch et al., 1978; La Barre, 1984; La Barre et al., 1986). Other studies have shown that terpenes are involved in competitive interactions between soft corals and their neighbors. Thus, soft corals have been shown to exert allelopathic effects on neighboring scleractinian corals (Sammarco et al., 1983), and soft coral terpenes have been detected in seawater around soft corals (Coll et al., 1982a) and have been shown to kill scleractinian corals at 5–10 ppm (Coll and Sammarco, 1983). The important roles played by secondary metabolites in the survival strategies of mature colonies of soft corals has thus been established. It seemed logical to carry this research forward into aspects of soft coral reproduction.

Soft corals are colonial marine invertebrates that reproduce both asexually by budding and colony division and sexually (Bayer, 1973). Sexual reproduction in the octocorallia proceeds via a number of different strategies including broadcast spawning [e.g., *Alcyonium digitatum* (Hartnoll, 1975), *Lobophytum* spp. (Yamasato et al., 1981; Bowden et al., 1985), *Sarcophyton* spp. (Benayahu and Loya, 1986), *Sinularia* spp. and *Cladiella* spp. (Alino et al., 1987)], surface brooding of fertilised eggs [e.g., *Clavularia* sp. (Alino, unpublished results), *Efflatounaria* sp. (Dinesen, 1985), *Capnella gaboensis* (Farrant, 1985), and *Parerythropodium fulvum fulvum* (Benayahu and Loya, 1983)], and by internal fertilization [e.g., *Xenia* spp. (Gohar, 1940; Benayahu and Loya, 1984, 1985)].

Because the pioneering work on the synchronized mass spawning of scleractinian corals on the Great Barrier Reef was carried out in Townsville (Harrison et al., 1984), we were able to collect and investigate the chemistry of the eggs spawned by soft corals, which occurred at approximately the same time (Harrison et al., 1984). A report on our investigation of chemical differences between the eggs spawned by *Lobophytum* spp. and the postspawning colonies has appeared (Bowden et al., 1985; Coll et al., 1987). We here report parallel chemical studies on the genus *Sinularia*, in which six colonies from two different sites in the Townsville region of tropical North Queensland were investigated over several spawning seasons.

METHODS AND MATERIALS

Collections

Australia. Techniques for the collection of eggs were developed during 1983 and eggs were collected from a *Sinularia* sp. (SM 83/1) during the spawning at Magnetic Island (146°51'E; 19°09'S) in October 1983. The modified plankton net used for the collection of the buoyant eggs has been described

(Bowden et al., 1985). A small sample of the soft coral colony was collected for chemical analysis several days after spawning, frozen, and freeze dried.

During spawning at Magnetic Island in November 1984, eggs were collected from a *Sinularia* sp. (SM 84/1), identical species to that studied in 1983, and portions of the colony were sampled four weeks before spawning, and one and 180 days after spawning. The tissue was frozen and freeze dried. Samples of the spawned eggs were frozen on collection and freeze dried prior to chemical analysis.

In Pioneer Bay on Orpheus Island (146°18'E; 19°09'S) in November 1984, a number of female colonies of *Sinularia* spp. (SM 84/6, SM 84/19, SM 84/20, SM 84/21) were sampled, and the tissue stored frozen and freeze dried for chemical assessment. During the spawning in December 1984, eggs were collected from these colonies, frozen, and freeze dried prior to extraction and chemical analysis.

Hawaii. Colonies of *Sinularia abrupta* Tixier-Durivault 1970 were collected off a pocket beach near the Blowhole, Oahu (157°50'W; 21°28'N) in 1973. Subsequent collections of *S. abrupta* from nearby sites were made in September 1984 (Moku Manu Island, Kaneohe Bay) and December 1984 (Blowhole and off Queen's Beach). Colonies were stored frozen.

Chemical Assessment

The freeze dried eggs and coral tissue from the Australian *Sinularia* colonies were each separately extracted with dichloromethane and the terpenoid components isolated from the crude extracts by chromatography on silica gel (see Bowden and Coll, 1986). Complete separations and quantitative and qualitative comparisons were performed using a Waters 6000A solvent delivery system for high-pressure liquid chromatography (HPLC) in conjunction with a Waters R401 refractive index detector, and two silica gel HPLC columns in series (Techsil 5 silica and Hewlett Packard Si-100 7 μ m) with ethyl acetate-light petroleum (35:65) as eluent. Structural assignments were initially made on the basis of [¹H]- and [¹³C]NMR comparisons with reported values. Other physical parameters (e.g., mp, [α]_D, etc.) unambiguously confirmed the identity of the major metabolites. Thin layer chromatography (TLC) was used initially to assess the absence or presence of metabolites in certain extracts. Frozen colonies of *Sinularia abrupta* from Hawaii were extracted with ethanol before chromatography, as reported elsewhere (Missakian et al., 1975).

X-Ray Crystallographic Determinations

Preliminary X-ray photographs of pukalide (from *S. abrupta*) (Missakian et al., 1975) showed orthorhombic symmetry. Accurate lattice parameters, determined from a least-squares fitting of 15 diffractometer measured 2θ values,

were $a = 9.048(1)$, $b = 12.562(1)$, and $c = 19.134(3)$ Å. Systematic extinctions, crystal density, and the presence of chirality were uniquely accommodated in space group $P2_12_12_1$ with one molecule of composition $C_{21}H_{24}O_6$ forming the asymmetric unit. All unique diffraction maxima with $2\theta \leq 114^\circ$ were collected using variable speed, 1° ω -scans and graphite monochromated Cu $K\alpha$ radiation (1.54178 Å). Of the 1516 reflections surveyed, 1444 (95%) were judged observed [$F_o > 3\sigma(F_o)$] after correction for Lorentz, polarization, and background effects.

The structure was solved routinely using the MULTAN system of programs (Main et al., 1980; Leonowicz, 1978; Hirotsu and Arnold, 1980; Hirotsu and Van Duyne, 1985; Motherwell, 1978). The first E synthesis revealed a plausible 18-atom fragment, which was completed using Fourier syntheses. Full-matrix least-squares refinements with anisotropic nonhydrogen atoms have converged to a standard crystallographic residual of 0.088 for the observed reflections.

Single-crystal X-ray diffraction analysis of epoxy-pukalide (from *Placogorgia* sp.) (Li, 1986) was performed. Lovely multifaceted crystals of epoxy-pukalide were grown from ethanol saturated with water. Preliminary X-ray photographs displayed orthorhombic symmetry, and accurate lattice constants of $a = 12.372(2)$, $b = 9.129(1)$, and $c = 17.097(2)$ Å were obtained from a least-squares fit of 15 diffractometer measured 2θ values. The systematic extinctions, crystal density, and presence of chirality were uniquely accommodated by space group $P2_12_12_1$ with one molecule of composition $C_{21}H_{24}O_7$ forming the asymmetric unit. All unique diffraction maxima with $2\theta \leq 114^\circ$ were collected on a computer-controlled four-circle diffractometer using graphite monochromated Cu $K\alpha$ radiation (1.54178 Å) and variable speed 1° ω -scan. After correction for Lorentz, polarization, and background effects, 1317 (86%) of the 1532 unique reflections were judged observed and used in subsequent calculations. A phasing model was obtained from a multiresolution tangent formula approach, and a partial molecular model was extended by weighted Fourier techniques (Main et al., 1980; Leonowicz, 1978; Hirotsu and Arnold, 1980; Hirotsu and Van Duyne, 1985; Motherwell, 1978). After partial least-squares refinements, the hydrogen atoms were located in a difference electron density synthesis. Further refinement with anisotropic nonhydrogen atoms and isotropic hydrogens have converged to a standard crystallographic residual of 0.060 for the observed reflections.

RESULTS

Chemical analysis of a postspawning sample of soft coral and of the eggs released from the same coral (*Sinularia* sp., SM 83/1) in November 1983 revealed significant chemical differences. The major metabolites in the egg

extracts were pukalide and the then unreported diterpene epoxy-pukalide. The coral tissue contained none of these diterpenes. It was initially assumed that the spawning samples had become confused, as neither pukalide nor epoxy-pukalide were present in the postspawning sample of SM 83/1. The reliability of this early result was confirmed during the 1984 spawning (see Table 1).

Table 1 summarizes the comparative analyses of eggs and coral tissue from the five *Sinularia* soft corals collected near Townsville. The analyses were performed to determine the presence or absence of pukalide and epoxy-pukalide; the nature of other terpenoids present in the soft corals is not reported. Subsequent analysis of these and other *Sinularia* colonies during the 1986 and 1987 spawnings revealed the reproducibility of these findings (Heaton, 1988).

Chemical analysis of colonies of *Sinularia abrupta* collected near the Blowhole, Oahu, in 1973 afforded pukalide in 0.01% yield (Missakian et al., 1975). Subsequent collections between 1975 and the present, including the collections of several morphologically different colony types of *S. abrupta* from a number of sites in 1984, failed to afford pukalide.

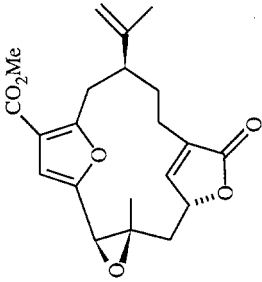
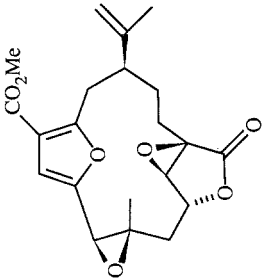
In the course of these chemical investigations, the single crystal X-ray structure determinations of pukalide (Missakian et al., 1975) and epoxy-pukalide (Li, 1986) were obtained and the ORTEP views of these molecules so determined appear in Figure 1. Tables 2 and 3 list the fractional coordinates of pukalide and epoxy-pukalide.¹

DISCUSSION

Our studies of the chemical composition of eggs spawned by soft corals reveal significant differences between the terpenoid composition of the eggs and the terpenoid composition of the pre- and postspawning colonies. Thus *Lobophytum compactum* eggs contained significant amounts of the diterpene thunbergol while this compound was not present in pre- or postspawning colonies. By contrast, *L. crassum* eggs contained none of the major terpenoid 13-hydroxylobolide component present in the pre- and postspawning colony (Bowden et al., 1985). Again, the eggs of *L. microlobulatum* contain the diterpene decaryiol and its precursor 3,4-epoxynephtenol, whereas the coral tissue contained only decaryiol. 3,4-Epoxynephtenol was not detected in the coral tissue from which the eggs were released (Coll et al., 1986). Clearly, the chemical composition of soft coral eggs is not just a random deposition of the usual soft coral terpenes in the eggs. It appears to be specific with respect to both content and timing. Furthermore, each *Lobophytum* species appears to have different diterpenes specific to the ovulation process.

¹ Complete crystallographic tables, including calculated and observed structure factors and thermal parameters, have been deposited with the Cambridge Crystallographic Centre: Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge CBL 1EW U.K.

TABLE 1. CHEMICAL ANALYSIS OF SEVERAL SIMILIARIAN SOFT CORAL COLONIES AND EGGS RELEASED THEREFROM^a

Similarian colony and species	Quantity extracted	Metabolite	Yield (mg)	Yield (mg)
SM83/1 [<i>S. gibberosa</i> (Tixier-Durivault)] Postspawning colony (+1 weeks) October spawned eggs	130 g	 <p>pukaide</p>	undetected	undetected
	2.4 g		23	14
	SM84/1 [<i>S. gibberosa</i> (Tixier-Durivault)] Pre spawning colony (-3 weeks) November spawned eggs Post spawning colony (+1 day) Post spawning colony (+6 months)	3 g	 <p>epoxy pukaide</p>	undetected
3 g		35		35
32 g		13		undetected
3 g		undetected		undetected

SO84/6 [<i>S. pavidus</i> (Tixier-Durivault)]				
Prespawning colony (-4 weeks)	37 g	undetected	undetected	undetected
December spawned eggs	15 g	120	16	16
SO84/19 [<i>S. polydactyla</i> (Ehrenberg)]				
Prespawning colony (-4 weeks)	TLC	undetected	undetected	undetected
December spawned eggs	17 g	330	undetected	undetected
SO84/20 [<i>S. rigida</i> (Dana)]				
Prespawning colony (-4 weeks)	TLC	undetected	undetected	undetected
December spawned eggs	14 g	150	60	60
SO84/21 [<i>S. conferta</i> (Tixier-Durivault)]				
Prespawning colony (-4 weeks)	TLC	undetected	undetected	undetected
December spawned eggs	17.5 g	300	130	130

^aIsolated yields are reported; qualitative absences (t/c, <0.01%) are described as "undetected."

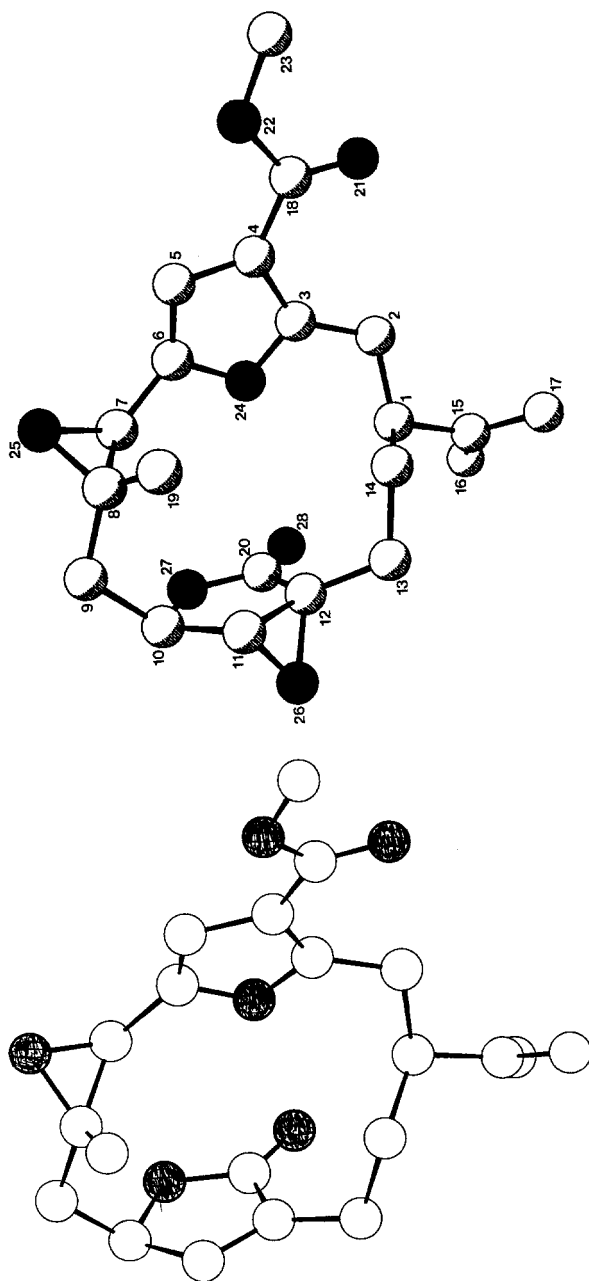


FIG. 1. Computer-generated perspective drawings of the final X-ray models of pukalide and epoxykukalide. Hydrogens are omitted for clarity and no absolute configuration is implied.

TABLE 2. FRACTIONAL COORDINATES AND THERMAL PARAMETERS FOR PUKALIDE^a

Atom	x	y	z
C(1)	0.6733(7)	-0.0580(4)	0.5177(3)
C(2)	0.5992(8)	-0.0363(5)	0.4380(4)
C(3)	0.4586(7)	0.0269(4)	0.4450(3)
C(4)	0.3799(7)	0.0915(5)	0.3980(4)
C(5)	0.2583(8)	0.1305(5)	0.4438(4)
C(6)	0.2671(7)	0.0825(6)	0.5139(4)
C(7)	0.1979(8)	0.0916(6)	0.5943(4)
C(8)	0.2372(8)	0.1792(5)	0.6477(4)
C(9)	0.2437(9)	0.1600(5)	0.7357(5)
C(10)	0.3764(9)	0.0952(6)	0.7627(4)
C(11)	0.5242(9)	0.1256(6)	0.7303(4)
C(12)	0.5877(8)	0.0404(5)	0.6946(4)
C(13)	0.7326(7)	0.0356(5)	0.6509(4)
C(14)	0.7190(7)	0.0469(5)	0.5612(4)
C(15)	0.8034(10)	-0.1327(6)	0.5075(4)
C(16)	0.7949(12)	-0.2276(7)	0.5428(9)
C(17)	0.9223(12)	-0.1075(13)	0.4575(9)
C(18)	0.4226(7)	0.1201(6)	0.3163(4)
C(19)	0.3295(11)	0.2737(6)	0.6169(5)
C(20)	0.4849(9)	-0.0483(6)	0.7040(4)
O(21)	0.5201(8)	0.0766(5)	0.2801(3)
O(22)	0.3407(5)	0.1979(4)	0.2869(2)
C(23)	0.3690(10)	0.2267(7)	0.2056(4)
O(24)	0.3874(5)	0.0173(3)	0.5166(2)
O(25)	0.0968(6)	0.1762(5)	0.6091(3)
O(26)	0.3580(6)	-0.0157(4)	0.7410(3)
O(27)	0.5016(7)	-0.1420(4)	0.6839(3)
H(1)	0.6180(70)	-0.1140(50)	0.5510(40)
H(2A)	0.6590(70)	0.0040(50)	0.3990(30)
H(2B)	0.5760(70)	-0.1070(50)	0.4150(30)
H(5)	0.1770(70)	0.1910(50)	0.4230(40)
H(7)	0.2120(70)	0.0110(50)	0.6230(30)
H(9A)	0.2630(70)	0.2380(50)	0.7630(40)
H(9B)	0.1590(70)	0.1190(50)	0.7570(40)
H(10)	0.3780(70)	0.0820(50)	0.8300(40)
H(11)	0.5760(70)	0.1960(50)	0.7400(40)
H(13A)	0.8000(70)	0.0870(50)	0.6710(40)
H(13B)	0.7960(70)	-0.0390(50)	0.6630(30)
H(14A)	0.6490(70)	0.1030(50)	0.5560(40)
H(14B)	0.8050(70)	0.0640(50)	0.5380(30)
H(16A)	0.7070(70)	-0.2440(50)	0.5740(30)
H(16B)	0.8600(70)	-0.2900(50)	0.5450(30)
H(17A)	0.8750(70)	-0.1050(50)	0.3970(40)
H(17B)	0.9970(70)	-0.1800(50)	0.4570(40)
H(17C)	0.9530(70)	-0.0360(50)	0.4820(30)

TABLE 2. Continued

Atom	x	y	z
H(19A)	0.2980(70)	0.3330(50)	0.6470(40)
H(19B)	0.3200(70)	0.2930(5)	0.5630(40)
H(19C)	0.4310(70)	0.2620(50)	0.6340(40)
H(23A)	0.4750(70)	0.2500(50)	0.2010(30)
H(23B)	0.2900(70)	0.2890(50)	0.1920(40)
H(23C)	0.3370(70)	0.1670(50)	0.1700(40)

^aEstimated standard deviations of the least significant figures are given in parentheses.

TABLE 3. FRACTIONAL COORDINATES AND THERMAL PARAMETERS FOR EPOXYPUKALIDE^a

Atom	x	y	z	B
C1	0.3062(4)	0.0747(6)	0.7287(3)	5.2(2)*
C2	0.2836(4)	0.1423(6)	0.8091(3)	5.5(3)*
C3	0.2269(4)	0.2821(7)	0.8044(3)	5.2(2)*
C4	0.1576(4)	0.3601(6)	0.8531(3)	4.9(2)*
C5	0.1181(5)	0.4837(7)	0.8076(4)	6.0(3)*
C6	0.1647(4)	0.4737(6)	0.7379(3)	5.0(2)*
C7	0.1538(5)	0.5403(6)	0.6590(4)	5.9(3)*
C8	0.0677(4)	0.4927(6)	0.6066(3)	5.8(3)*
C9	0.0866(5)	0.4966(7)	0.5197(3)	6.3(3)*
C10	0.1564(5)	0.3759(7)	0.4869(3)	6.3(3)*
C11	0.1289(4)	0.2149(7)	0.5040(4)	5.7(3)*
C12	0.2194(4)	0.1476(6)	0.5445(3)	5.4(2)*
C13	0.2186(4)	0.0121(6)	0.5963(3)	5.5(2)*
C14	0.2024(4)	0.0402(6)	0.6845(3)	5.5(2)*
C15	0.3789(5)	-0.0610(7)	0.7358(4)	6.3(3)*
C16	0.4839(6)	-0.0497(12)	0.7096(6)	10.5(6)*
C17	0.3364(7)	-0.1897(8)	0.7705(6)	9.0(5)*
C18	0.1330(4)	0.3180(7)	0.9326(3)	5.7(3)*
C19	-0.0183(5)	0.3861(8)	0.6343(4)	6.8(3)*
C20	0.3043(4)	0.2619(7)	0.5474(3)	5.6(3)*
O21	0.1729(4)	0.2176(5)	0.9684(2)	7.3(2)*
O22	0.0535(3)	0.4009(4)	0.9632(2)	5.7(2)*
C23	0.0251(5)	0.3696(8)	1.0426(3)	7.0(3)*
O24	0.2334(3)	0.3552(4)	0.7346(2)	5.2(2)*
O25	0.0612(3)	0.6320(5)	0.6447(3)	6.9(2)*
O26	0.1993(4)	0.1210(5)	0.4623(2)	7.0(2)*
O27	0.2674(3)	0.3884(5)	0.5142(2)	6.6(2)*
O28	0.3934(3)	0.2527(5)	0.5741(3)	7.1(2)*
H1	0.347(4)	0.131(6)	0.697(3)	5.9(13)
H2A	0.246(4)	0.074(6)	0.848(3)	6.9(13)

TABLE 3. Continued

Atom	x	y	z	B
H2B	0.343(4)	0.148(6)	0.839(3)	6.9(14)
H5	0.169(4)	0.482(7)	0.837(3)	6.9(13)
H7	0.227(4)	0.574(6)	0.631(3)	7.3(14)
H9A	0.020(4)	0.506(7)	0.496(4)	7.7(14)
H9B	0.129(4)	0.585(7)	0.506(4)	7.7(14)
H10	0.161(4)	0.385(7)	0.427(4)	7.9(16)
H11	0.056(4)	0.192(6)	0.503(4)	6.8(13)
H13A	0.166(4)	-0.059(6)	0.580(3)	6.6(13)
H13B	0.295(4)	-0.047(6)	0.589(3)	6.6(13)
H14A	0.151(4)	0.126(6)	0.688(3)	6.6(13)
H14B	0.183(4)	-0.065(6)	0.698(3)	6.6(13)
H16A	0.471(6)	-0.040(10)	0.762(5)	12.8(25)
H16B	0.558(5)	-0.011(11)	0.685(6)	12.8(25)
H17A	0.297(5)	-0.224(9)	0.770(4)	10.9(20)
H17B	0.364(5)	-0.276(9)	0.774(4)	10.9(21)
H17C	0.395(6)	-0.163(9)	0.774(4)	10.9(20)
H19A	-0.041(4)	0.391(8)	0.679(4)	8.2(16)
H19B	-0.079(4)	0.406(7)	0.616(4)	8.2(16)
H19C	-0.000(4)	0.286(7)	0.631(3)	8.2(15)
H23A	-0.036(4)	0.374(7)	1.043(3)	7.9(15)
H23B	0.070(4)	0.396(7)	1.065(4)	7.9(16)
H23C	-0.000(4)	0.262(7)	1.048(3)	7.9(15)

^aStandard deviations of the least significant figures are given in parentheses. The isotropic equivalent thermal parameter is given for anisotropic atoms (denoted by an asterisk).

The results depicted in Table 1 show that, in the case of a number of *Simularia* species, the terpenes epoxypukalide and/or pukalide are present only in the eggs and are probably formed during the month prior to spawning. This implies that these compounds are synthesized within the eggs and may have some function. A number of possible roles may be considered. It is possible that pukalide and epoxypukalide play a defensive role, either in relation to fish predation or in relation to pathogens in the water column, which may use the fertilized eggs and planulae as a food source. The former suggestion does not appear to be correct, for on the night of spawning, colonies releasing eggs can be identified by the presence of a cloud of small fish above the colony. The fish eat large quantities of eggs with no obvious ill effects (J. Coll, personal observations). The possibility that the compounds may be effective in protecting the planktonic (planula) phase of the corals has not been borne out by subsequent microbiological study (P. Alino, unpublished results).

In a dioecious broadcast spawning coral, some chemicals in the eggs must

serve a role in chemotaxis, i.e., they must attract sperm to the released eggs. We have recently implicated lipophilic molecules in this role for a *Lobophytum* species (Coll et al., 1987). It is possible that pukalide and epoxy-pukalide may play such a role, although their nature, and the quantities present in the eggs seem to be out of character with known chemotactic substances (Moore, 1977). Usually the amounts are much less than those reported here. Further investigation of this phenomenon is scheduled for the next annual spawning.

Yet another role possible for pukalide and epoxy-pukalide may be as a chemical release factor for ovulation. In the final stages of egg production, it is possible that these compounds are biosynthesized within the eggs until a threshold concentration is reached, at which time egg release occurs. Steroidal hormones play this type of role in animals; it is not impossible that terpenes like pukalide might play this role in invertebrates. Many other specific roles might be suggested: these include chemicals that prevent hybrid formation and act as muscle relaxants on the polyp to ease the release of eggs from the polyp, or as a juvenile hormone.

Pukalide and pukalide-like compounds are widespread among the Octocorallia (see Faulkner, 1984, 1986). They may be among the most highly oxygenated terpenes known. Lophotoxin, isolated together with pukalide, in 0.2% dry weight from *Lophogorgia rigida*, is certainly among the most toxic (Fenical et al., 1981). The isolation of trace amounts of pukalide from *Lophogorgia rigida* (Fenical et al., 1981), of trace amounts of epoxy-pukalide from *Leptogorgia setacea* (Ksebati et al., 1984) and from the deep sea gorgonian *Placogorgia* sp. (Li, 1986), and the inability to re-isolate pukalide (1) from *Sinularia abrupta* (Klein, 1986) may all be explained on the basis of the involvement of pukalide and epoxy-pukalide in the spawning of a large number of different soft corals. It seems likely that many of these isolations were fortuitous, i.e., that the initial collections coincided with the spawning season.

Structural elucidation of pukalide, epoxy-pukalide, and lophotoxin has been based on spectroscopic deduction and chemical interconversion. Because of the interdependence of these structural assignments and the availability of two as yet unpublished X-ray crystallographic determinations, it was decided to publish the structures in this report. The molecules now appear to be common components in a large number of octocorallian systems; it is essential that their structures be secure and their stereochemical parameters completely defined. Fortunately, both the original structure proposed for pukalide (Missakian et al., 1975) and for epoxy-pukalide (Ksebati et al., 1984) were correct, but it is important that all researchers in this area have the confidence of X-ray structures for these apparently important molecules.

At this point in time, the exact function of pukalide and epoxy-pukalide remains a mystery. Neither compound appears to be particularly toxic or antibiotic. They may yet be shown to be chemotactic substances or to play a role

in ovulation. Their three-dimensional structures and physical properties are secure, and they are available for testing; future research will seek to define their function. Unlike many other natural products, these compounds appear to be present in the majority of species of the genus *Sinularia* and only present in the eggs of these corals during the month prior to ovulation. In this sense, they are clearly distinguished from the majority of natural products.

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ALLELOPATHY, CHEMICAL COMMUNICATION, AND PLANT DEFENSE

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Abstract—Allelopathy is identified particularly with chemical activity between plants; entomologists refer to allelochemicals in a broader context. Recent work shows that several groups of compounds associated with allelopathy also play a part in communication between plants and other organisms. It is argued that such communication is part of the similarities in plant and animal responses to stress and may contribute to plant defense.

Key Words—Allelopathy, allelochemicals, mitochondria, phagocytosis.

INTRODUCTION

Chemicals are frequently employed in the defense systems of animals, even when physical defenses are well developed (Cogger, 1979). Plants also have physical adaptations for defense and produce a wide spectrum of secondary chemical compounds (Levin, 1976), some of which are active in allelopathy. Several compounds associated primarily with allelopathy, defined as biochemical interactions between plants (including microorganisms traditionally placed in the plant kingdom), appear to have a wider role in plant self-defense (Lovett, 1982a).

Reese (1979) uses the term "allelochemical" to describe "nonnutritional chemicals produced by one organism that affect the growth, health, behavior or population biology of other species". Biological reaction to such compounds characteristically is one of attraction or stimulation at low concentrations, with the response becoming increasingly one of repulsion or inhibition as the con-

centration increases. The phenomenon of "hormoligosis" (Luckey, 1968), in which toxic insecticides at very low concentrations act as growth stimulants, is analogous.

Plants respond in this manner to many organic and some inorganic chemicals, for example, aluminium (Bennet et al., 1987). Chemicals developed for management of other organisms, for example, insecticides (Chapman and Allen, 1948), will sometimes elicit this type of response in plants.

Monoterpenoids are one group of natural compounds for which such responses have been documented in a range of organisms. Examples include insect larvae (Selander et al., 1974); bacteria and a yeast (Andrews et al., 1980); feeding behavior of mammals (Sheehy and Winward, 1981; Welch and McArthur, 1981; White et al., 1982); and fungal growth (Franich et al., 1982). Similar compounds are allelopathic (Muller, 1966).

Allelochemicals may, therefore, be considered as part of a network of communication in which disparate organisms give similar responses to similar compounds or families of compounds. Plants producing biologically active compounds at relatively high concentrations may be perceived as utilizing chemical defenses.

Plant allelochemicals frequently induce stress in other plants. Such stress may contribute to interference between plants; again, a dimension of defense. We report here on observations of responses to allelopathic stress in some plant species and identify further similarities with responses of other organisms to allelochemicals.

METHODS AND MATERIALS

Linum usitatissimum L. (linseed) seedlings were grown in bioassay (Lovett and Duffield, 1981) over a dilution series of the allelochemical benzylamine, produced in leaf washings of the cruciferous weed *Camelina sativa* L. Crantz (Camelina) (Lovett and Duffield, 1981), with sterile water as the control. Seedlings of *Sinapis alba* L. (white mustard) were grown under similar conditions and exposed to a dilution series of the allelochemicals gramine and hordenine, produced by *Hordeum vulgare* L. (barley) (Overland, 1966). Radicle length of all seedlings was measured after five days of incubation at 24°C in the dark.

The methods of Jones and Varner (1967) and Reeve and Crozier (1975) were used to assess the release and activity of α -amylase in barley, as described by Lovett and Potts (1987).

Thin sections of linseed and white mustard root tips for electron microscopic examination were prepared using a modification of the technique of Lorber and Muller (1976) as described by Levitt et al. (1984).

RESULTS AND DISCUSSION

Radicle length of seedlings of *Linum usitatissimum* L. (linseed) showed the characteristic response to the allelochemical benzylamine (Figure 1). *Sinapis alba* L. (white mustard) showed a similar response to the alkaloids gramine and hordenine produced by *Hordeum vulgare* L. (barley) during germination (Figures 2 and 3).

These responses may be termed secondary effects (*sensu* Winter, 1961) as they reflect primary metabolic disruptions. Interference with the ability of germinating seedlings of linseed (Lovett, 1982b) and *Helianthus annuus* L. (sunflower) (Levitt et al., 1984) to use food reserves has been suggested as an example of such disruption. Reese (personal communication) notes that inhibition of efficiency in converting ingested food is a response of insects to ingested allelochemicals. An example is the response of premature weevils to α -terpineol (Figure 4). In these data of Selander et al. (1974) we note that it is the juvenile form that, like young seedlings, may be most susceptible to biologically active chemicals.

Impaired enzyme activity, identified by Rice (1984) as one primary target for allelopathic activity, may explain a reduced ability to metabolize food reserves such as starch in plants. Recent data indicate that the response of α -amylase, involved in the breakdown of starch, to the allelochemical scopolamine (Lovett et al., 1981) is similar to that observed for gross morphological characteristics such as radicle length (Figure 5). Roshchina et al. (1986) reported

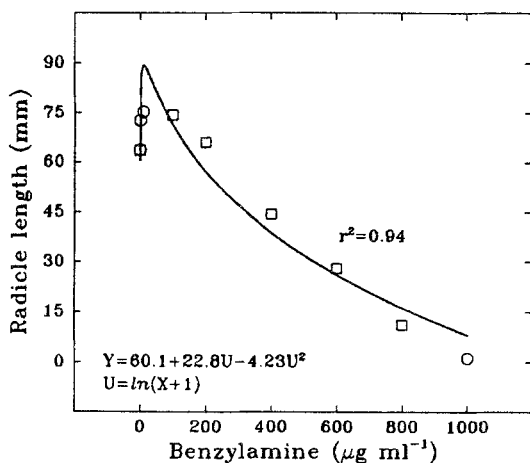


FIG. 1. The response in radicle length of linseed to benzylamine (data from Lovett and Duffield, 1981).

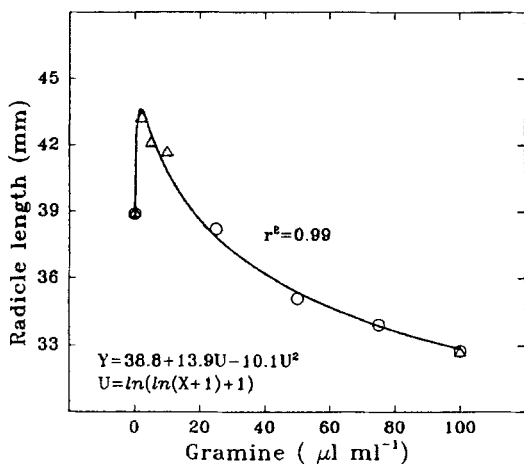


FIG. 2. The response in radicle length of white mustard to gramine (data from Liu and Lovett, 1987).

a similar effect of the alkaloid capsaicine on photosynthetic electron transport in isolated pea chloroplasts. Thus, responses in radicle length or other gross morphological parameters may be explained in terms of similar responses at the level of the cell.

Transmission electron microscopy of linseed and white mustard root tips showed a similar response to allelopathic stress in the root tip cells (Figures 6–

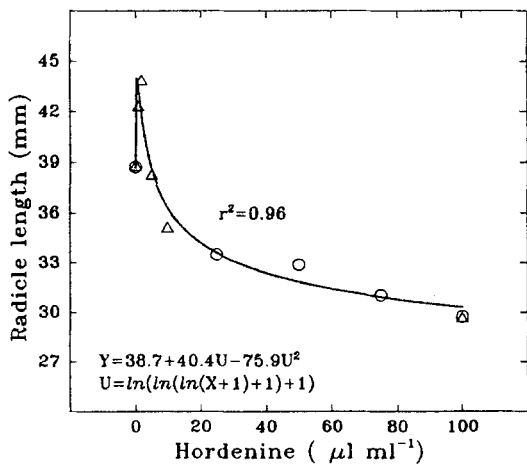


FIG. 3. The response in radicle length of white mustard to hordenine (data from Liu and Lovett, 1987).

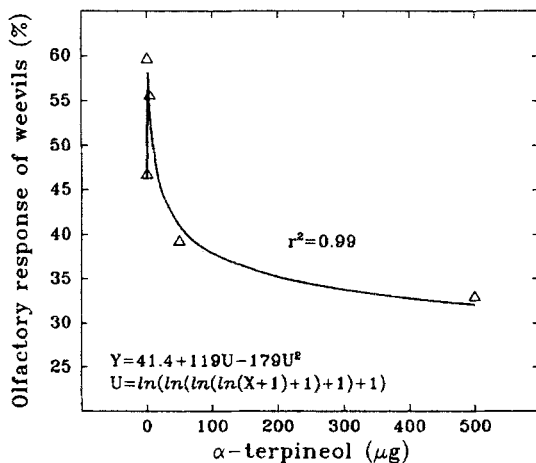


FIG. 4. Olfactory response of weevil larvae to α -terpineol (data from Selander et al., 1974).

8). While control cells showed distinct nuclei, intact organelles, and some small vacuoles (Figures 6 and 8a), increasing concentration of allelochemicals brought about increases in number and size of vacuoles and evidence for phagocytosis (Figures 7 and 8b). Nuclei became less distinct as the concentration of allelochemicals increased and some mitochondria showed evidence of disorganization (Figures 7b-7d and 8b).

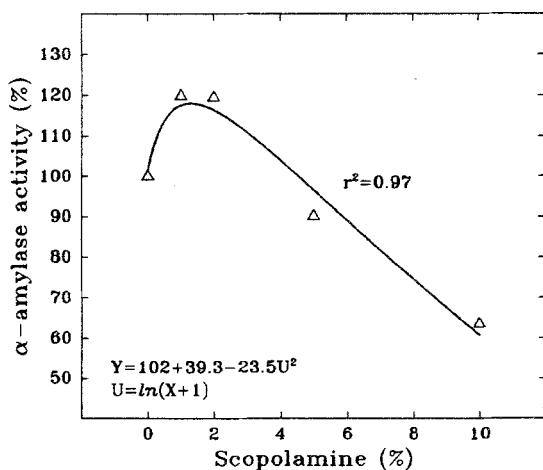


FIG. 5. The response of α -amylase activity to scopolamine.

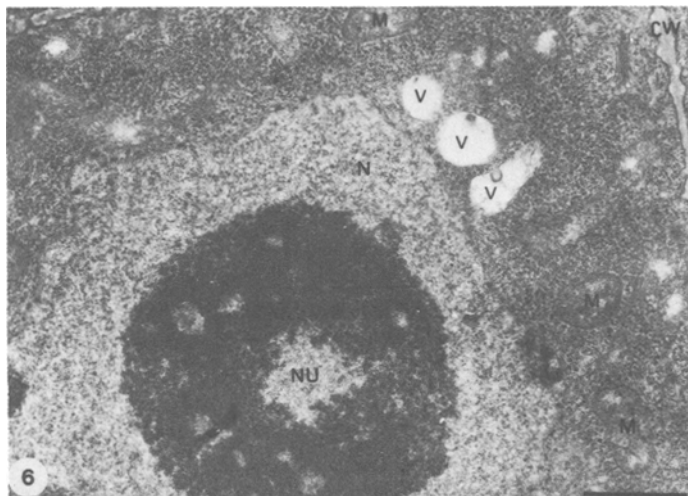


FIG. 6. Linseed root tip, sterile water control. Scale bar = 1 μ m. CW = cell wall; V = vacuole; M = mitochondrion; N = nucleus; NU = nucleolus.

Matile (1984) has discussed the detoxification of potentially toxic plant metabolites and highlights the vacuole as a compartment where these toxins may be sequestered. The consistent response of plant root tip cells to allelopathic stress, in which vacuolar development is prominent, shows similarities to those observed in response to other stresses. For example, similar responses occur following invasion by plant parasitic nematodes of root cells of *Apium graveolens* L. (celery) (Bleve-Zacheo et al., 1979) or exposure of *Allium cepa* L. (onion) to heavy metals such as lead (Wierzbicka, 1987). There are close similarities also between the plant vacuoles described here and, for example, autophagic vacuoles in mammals such as the rat (Ahlberg et al., 1982). The limits to which these analogies can be extended is the subject of continuing work in which the roles and relationships of vacuoles, vesicles, endoplasmic reticulum, Golgi apparatus, and mitochondria are being investigated (Lovett et al., 1987; Lovett and Ryuntyu, 1988).

While several metabolic pathways are common to plants and animals, plants differ from animals in important respects, such as lacking a nervous system. We suggest that the evidence presented here points to allelochemicals as important communicators between plants and other organisms. Allelochemicals may attract or repel organisms associated with plants and may contribute to plant

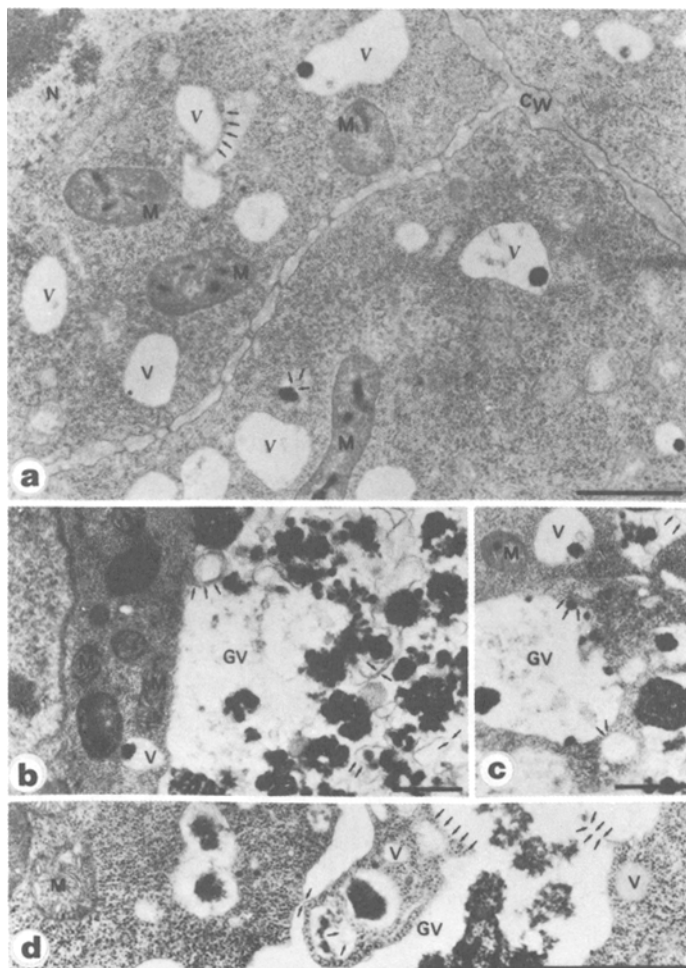


FIG. 7. Linseed root tip, 100 $\mu\text{g/liter}$ benzylamine applied. (a). Linseed root tip, 1000 $\mu\text{g/liter}$ benzylamine applied (b-d). Scale bar = 1 μm . CW = cell wall; V = vacuole; GV = giant vacuole; M = mitochondrion; N = nucleus; arrows indicate phagocytosis.

defense under stress, a role analogous to that of chemicals produced by animals (Edmunds, 1974).

Matile (1975) has drawn attention to the similarities of some lytic phenomena in plants and animals. Autophagic vacuoles are one example and may also

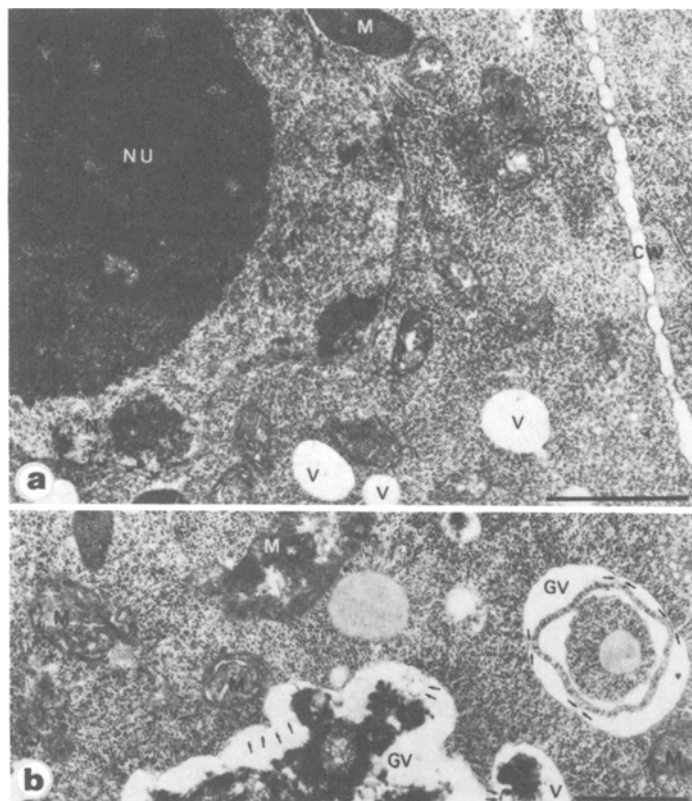


FIG. 8. White mustard root tip, sterile water control (a). White mustard root tip, 100 $\mu\text{g/liter}$ hordenine applied (b). Scale bar = 1 μm . CW = cell wall; V = vacuole; GV = giant vacuole; M = mitochondrion; NU = nucleolus; arrows indicate phagocytosis.

be part of a common response to stress, suggesting that further analogies will become apparent as the combined disciplines of morphology, cytology, and biochemistry are brought to bear on allelopathic phenomena.

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BIOCHEMICAL INVESTIGATIONS OF ANTIBIOSIS MATERIAL IN LEAF EXUDATE OF WILD *Nicotiana* SPECIES AND INTERSPECIFIC HYBRIDS

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Abstract—A model system involving several *Nicotiana* species containing novel nicotine alkaloids was used to study heritability and expression of alkaloid production in leaf trichomes. The three species that comprise the section Repandae (*N. repanda*, *N. stocktonii*, and *N. nesophila*) were hybridized with either *N. tabacum* or *N. sylvestris* (neither of which produces *N*-acynornicotine). The progeny of the hybrid with *sylvestris* produced the *N*-acynornicotines at a level found in the Repandae parent. *Nicotiana repanda* was crossed to *N. tabacum*, and the F₂ progeny produced the alkaloid at the same level as the original Repandae parent. Inheritance of the ability to acylate normicotine in Repandae species is inherited in hybrids in a dominant manner. These and other data obtained suggest that the *N*-acyltransferase that acylates normicotine in Repandae species inherited in hybrids is in a dominant manner and that the regulatory sequence(s) for the gene is expressed in leaf trichomes when the gene is in a foreign *Nicotiana* background.

Key Words—*Nicotiana* sp., Repandae, trichomes, nicotine, alkaloids, *Manduca sexta*, Lepidoptera, Sphingidae, acynornicotines.

INTRODUCTION

Host-plant resistance has long been recognized as an attractive and viable pest defense strategy (Painter, 1951; Kogan, 1982; Maxwell and Jennings, 1980). The most conventional approach to accessing new forms of insect resistance has involved intra- and interspecific hybridizations, but crossing barriers may prevent access to potentially effective forms of resistance in related species. Several approaches for gaining access to resistance in sexually incompatible species are now available. These include use of bridging species, plant tissue culture procedures, and transfer of cloned genes. We are not aware of examples of commercial exploitation of any of these three approaches for suppression of insect attack. Further, there are no examples whereby a given insect resistance trait was moved into a commercial cultivar background in independent applications of each of these approaches. Thus, it is difficult to assess the individual or combined potential of these three approaches. Such a comparative study would be of considerable theoretical and practical interest.

Perhaps one reason for the lack of progress in this area is that such a comparative study would require the combined efforts of plant geneticists, insect physiologists, and organic chemists to provide firm documentation that the given antibiosis chemical is heritable and is expressed at levels effective against the target insect.

Cultivated tobacco, *Nicotiana tabacum* (L.), is protected from insect attack by nicotinoid alkaloids, which have been the mainstay of previous approaches to breeding for insect resistance (Burk and Chaplin, 1976). However, one lepidopteran pest of tobacco, *Manduca sexta* (L.), is resistant to nicotine through an effective combination of target site insensitivity and nicotine excretion mechanisms (Self et al., 1964; Morris, 1984). We have found that a group of wild *Nicotiana* species (section Repandae, comprised of species *repanda*, *stocktonii*, and *nesophila*), which is highly resistant to the hornworm, produces a group of novel nicotinoid alkaloids, *N*-acylnornicotines (NacylNN), which is extremely toxic to this insect (Jones et al., 1985; Huesing and Jones, 1987; Severson et al., 1985b, 1987). This alkaloid group, which is very easily detected by thin-layer chromatography (TLC), results from a single, terminal enzymatic addition to nornicotine (Zador and Jones, 1986), a substrate found in commercial tobacco (Severson et al. 1985a).

The *Manduca sexta*-*Nicotiana* system is attractive as a model system for testing new concepts and various approaches to gene transfer for insect resistance. This insect-plant system has the following attractive features: (1) a highly effective antibiosis chemical in wild plant hosts; (2) a structurally defined antibiosis factor; (3) an antibiosis chemical whose expression is easily scorable; (4) an antibiosis chemical that is a synthetic extension of one of the best characterized biochemical pathways of plant secondary product metabolism, the nicotine pathway; (5) an antibiosis chemical, which, being derived by a single

TABLE 1. SOURCE OF HYBRIDS USED IN ANTIBIOSIS STUDIES

Interspecific hybrid combination	Generation tested ^a	Reference
I. 4n(<i>N. repanda</i> × <i>N. sylvestris</i>)	S ₂	Burk, 1967
IIA. ((4n <i>N. repanda</i> × <i>N. tabacum</i> cv. SC 72) × <i>N. tabacum</i> cv. NC 95) × <i>N. tabacum</i> cv. Md 609	BC ₃ F ₁	Pittarelli and Stavely, 1975; Pittarelli and DeJong, 1988
IIB. 6n(4n <i>N. repanda</i> × <i>N. tabacum</i> cv. Md 872)	F ₂	Pittarelli, 1987 (personal communication)
III. 4n(<i>N. stocktonii</i> × <i>N. tabacum</i>) × <i>N. tabacum</i> cv. Burley 21	BC ₁ F ₁	Reed and Collins, 1978
IV. 4n(<i>N. nesophila</i> × <i>N. tabacum</i>) × <i>N. tabacum</i> cv. Burley 21	BC ₁ F ₁	Reed and Collins, 1978
V. 4n(<i>N. gossei</i> × <i>N. tabacum</i> cv. Fla 17)	S ₁	Burk and Dean, 1975
VIA. 4n(<i>N. benthamiana</i> × <i>N. tabacum</i>) [= C1] × <i>N. tabacum</i> cv. SR1	BC ₁ F ₁	DeVerna et al., 1987
VIB. 4n(<i>N. benthamiana</i> × <i>N. tabacum</i>) [= C3] × <i>N. tabacum</i> cv. SR1	BC ₁ F ₁	DeVerna et al., 1987
VII. (<i>N. stocktonii</i> or <i>N. nesophila</i> , <i>N. tabacum</i>) × <i>N. tabacum</i>	BC ₁ F ₁ or BC ₂ F ₁	DeVerna et al., 1987

^aS₂ = hybrid plants selfed for two generations; cv = cultivar; C1 and C3 are code names for the given hybrid.

enzymatic addition to a terminal pathway, is likely to be inherited as a simple genetic trait, facilitating genetic analysis; and (6) the enzyme which produces this antibiosis chemical is currently being purified for future genetic engineering experiments (Jones et al., 1987), facilitating direct comparison of molecular biology and the above-described gene transfer approaches. Furthermore, hybrids of Repandae species *N. tabacum* derived from (1) classical direct crossing (Pittarelli and Stavely, 1975), (2) use of bridging species (Burk, 1967), and (3) plant tissue culture (Reed and Collins, 1978) are currently available.

In these experiments we have applied plant genetics, insect physiology, and organic chemistry toward defining and documenting the successful transfer of *N*-acetylornicotine production into *N. tabacum*.

METHODS AND MATERIALS

Plant Material. The interspecific hybrid combinations investigated in this study are summarized in Table 1. Seed stocks were obtained from J.W. DeVerna, Campbell Institute for Research and Technology, Davis, California; G.W. Pittarelli, USDA-ARS, Beltsville, Maryland; S.M. Reed, N.C. State University, Raleigh, North Carolina; and V.A. Sisson, USDA-ARE, Oxford,

North Carolina. With the exception of hybrid combination IIB, the specific methods used to produce these hybrids have been reported previously and will only be summarized here. The first hybrid (I) is an interspecific bridge-cross originally designed to transfer disease-resistant germplasm for a donor species (*N. repanda*) through an intermediate or transfer species (*N. sylvestris*) into a recipient species (*N. tabacum*). In hybrid IIA an autotetraploid form of *N. repanda* (RR/RR) was crossed with diploid *N. tabacum* (TT) and the resulting sesquidiploid (RR/T) back-crossed to NC 95, and then three times to the tobacco cultivar Md 609. Hybrid IIB was produced in a similar manner except that the sesquidiploid (RR/T) was colchicine treated to produce a hexaploid plant (RRRR/TT), which was subsequently studied in the F₂ generation. Using fertilized ovule culture techniques, hybrids between *N. stocktonii* and *N. nesophila* with *N. tabacum* (III and IV) were achieved. Allotetraploid hybrids were obtained from callus culture of F₁ hybrid plants and the allotetraploids were back-crossed to diploid *N. tabacum*. Hybrid combination V resulted from a cross between *N. gossei* as the maternal parent with a Florida cigar-wrapper variety of *N. tabacum*. Plants of the initial sterile F₁ hybrid were treated with colchicine to produce a fertile allotetraploid. Finally, two different hybrids were generated using in vitro fertilization techniques to cross *N. benthamiana* with a *N. tabacum* parent. Allotetraploid derivatives, identified as C1 and C3, were each back-crossed to the breeding line SR1. *Nicotiana gossei* and *N. benthamiana* are members of the section *Suaveolentes*. These species have been shown to be resistant to *M. sexta*; however, the biochemical basis of their resistance is unknown. These crosses allow a comparison with the transfer of the defined resistance of the *Repandae* section.

Seed of all of these materials were germinated in Petri dishes in a 1-cm-deep Murashige and Skoog plant growth medium (Murashige and Skoog, 1962). After the seedlings had attained a height of ca. 1 cm, they were then transferred to 4 × 4-cm peat pots and raised to maturity in the greenhouse. When possible, both the wild parents and the cultivar were also planted to determine the pattern of alkaloid or resistance transfer.

Antibiosis toward M. sexta. Leaf material from the crosses with *N. repanda* was surface extracted at the vegetative (prebud) stage, separated by TLC, and stained with CNBr-paraaminobenzoic acid to determine if high amounts of surface alkaloids were present. A minimum of 15 leaves, one from each of 15 plants, was extracted. All leaves were chosen from the middle third of the plant. If the NacylNN was observed after staining, these plants were bioassayed with second instar *M. sexta* larvae by means of a four-day bioassay (Jones et al., 1985). In the case of the *N. benthamiana* material, all plants were bioassayed regardless of the TLC analysis. A minimum of 10 larvae were used in each bioassay, with the results being pooled for analysis.

Determination of NacylNN Content of Leaves. To determine the NacylNN content of the leaf exudate of the *Repandae* parents and their hybrids, six sam-

ples of 15 leaves were extracted and the extracts dried under vacuum. After weighing, a known quantity of exudate extract was obtained. Two 1-mg aliquots of each exudate extract were spotted to silica TLC, the plate developed, the appropriate region scraped and eluted, and the concentration of NacylNN determined spectrophotometrically. The amount of NacylNN per milligram of exudate and the amount per square centimeter of leaf surface were determined.

The quality and quantity of the common *Nicotiana* alkaloids nicotine, nor-nicotine, anabasine, anatabine, and *N*-formyl and *N*-acetyl nornicotine were analyzed on Superox capillary column with N-P detection, as described by Severson et al. (1984). The *N*-acyl and OH-NacylNN compounds were analyzed on a SE-54 capillary column after conversion of hydroxylated compounds to trimethylsilyl ethers. Components were characterized by GC retention and GC-MS data.

Statistical Analysis. Data on larvae scored in various categories during the experiment were converted to percentages for analysis. All repetitions of the bioassays were pooled for analysis. All categories are assumed to be mutually exclusive. Since decisions concerning which plant species to use in a breeding program will be based on whether the species is resistant and the magnitude of its resistance, confidence intervals were used in the analysis. Approximate 95% confidence limits for the difference between percentages were generated using the statistic, $|P_1 - P_2|$, $\pm t_{\alpha} \sqrt{[P_1(1 - P_1)/n] + [P_2(1 - P_2)/n]}$, with $\alpha = 0.05$. The occurrence of a type I error for a given inference on the magnitude of the difference between treatment effects will not affect the validity of inferences made on other treatment comparisons for the experiment. Therefore, we chose the *t* statistic for both its asymptotic power and amenability to a comparison-wise confidence interval structure, the latter being absent in some other procedures (Jones, 1984; Jones and Matloff, 1986). All treatments were compared to *N. tabacum* (KY 14), for analysis.

RESULTS

Section Repandae

All three species comprising this Repandae section (*N. nesophila*, *N. repanda*, and *N. stocktonii*) induced high levels of mortality, at least 80%, and adverse effects on both larval growth and feeding (Table 2). The confidence interval indicates that *N. repanda* produces a minimum 62% additional mortality above that of KY 14 (*N. tabacum*) (Table 3). In addition, none of the larvae fed heavily on the Repandae species, nor did any larvae reach the third instar.

The quantity of NacylNN per milligram of exudate in *N. repanda* was $83 \pm 4.5 \mu\text{g}$, or $10.7 \pm 0.58 \mu\text{g}/\text{cm}^2$ of leaf surface. The quality of the alkaloid was confirmed by GC as predominantly the β -hydroxy NacylNN (Table 4).

Nicotiana repanda X *N. sylvestris* (I). This hybrid caused high levels of

TABLE 2. EFFECTS OF WILD SPECIES AND HYBRIDS ON MORTALITY, FEEDING, AND GROWTH OF *M. sexta* LARVAE AFTER 4 DAYS OF EXPOSURE, EXPRESSED AS PERCENTAGES WITH 95% CONFIDENCE LIMITS

Species or cross	N	Percentages (95% confidence limits) ^a		
		Mortality	Heavy feeding ^b	Attain third instar
1. <i>N. tabacum</i> (KY 14)	20	5 (0, 15)	85 (68, 100)	100 (86, 100)
2. <i>N. sylvestris</i>	20	0 (0, 15)	100 (86, 100)	100 (86, 100)
3. <i>N. stocktonii</i>	40	100 (99, 100)	0 (0, 5)	0 (0, 5)
4. <i>N. nesophila</i>	10	80 (53, 100)	0 (0, 27)	0 (0, 27)
5. <i>N. repanda</i>	20	85 (68, 100)	0 (0, 15)	0 (0, 15)
6. <i>N. repanda</i> × <i>N. sylvestris</i>	25	72 (53, 91)	8 (0, 18)	0 (0, 13)
7. <i>N. repanda</i> × MD 872	23	74 (55, 93)	0 (0, 13)	0 (0, 13)
8. <i>N. repanda</i> × MD 609	54	9 (2, 16)	100 (79, 100)	100 (79, 100)
9. R 25	40	33 (14, 52)	83 (71, 95)	72 (53, 91)
10. <i>N. benthamiana</i>	21	71 (50, 92)	42 (19, 65)	24 (4, 44)
11. C1 × SR1	30	3 (0, 10)	97 (91, 100)	97 (91, 100)
12. C3 × SR1	18	6 (0, 18)	94 (92, 96)	94 (92, 96)
13. <i>N. gossei</i>	15	33 (12, 58)	60 (28, 92)	60 (29, 91)
14. <i>N. gossei</i> × FLA 17	42	2 (0, 6)	93 (85, 100)	43 (28, 58)

^a95% confidence limits (binomial statistic) are lower and upper, respectively.

^bMore than five bites taken.

TABLE 3. DIFFERENCE BETWEEN PERCENTAGES AND 95% CONFIDENCE LIMITS FOR THOSE DIFFERENCES, AFTER 4 DAYS OF EXPOSURE OF *M. sexta* LARVAE TO SELECTED SPECIES OR CROSSES OF *Nicotiana*.

Comparison	Percentages (95% confidence limits) ^a		
	Mortality	Feeding	Attain third instar
1. KY 14 vs. <i>N. repanda</i>	80 (62, 98)*	85 (69, 100)*	100 (90, 100)*
2. KY 14 vs. <i>N. repanda</i> × (MD872)	69 (49, 89)*	85 (69, 100)*	100 (90, 100)*
3. KY 14 vs. <i>N. rapanda</i> × <i>N. sylvestris</i>	67 (36, 98)*	77 (58, 96)*	100 (90, 100)*
4. KY 14 vs. <i>N. nesophila</i>	75 (48, 100)*	85 (69, 100)*	100 (90, 100)*
5. KY 14 vs. <i>N. stocktonii</i>	95 (85, 100)*	85 (69, 100)*	100 (90, 100)*
6. KY 14 vs. <i>N. benthamiana</i>	66 (44, 88)*	43 (16, 70)*	76 (55, 97)*
7. KY 14 vs. R25	28 (10, 46)*	2 (-18, 22)	28 (11, 45)*
8. KY 14 vs. <i>N. gossei</i>	28 (10, 46)*	25 (-5, 55)	40 (13, 67)*

^a95% confidence intervals (*t* statistic) that do not contain zero between upper and lower confidence limits (denoted by *) are equivalent to a hypothesis test in which $P < 0.05$. 95% confidence limits are lower and upper, respectively. For "mortality" and "feeding," the differences are the wild parent minus KY 14; for "attain third instar" the differences are KY 14 minus wild parent.

TABLE 4. DISTRIBUTION (PERCENTAGE BASIS) OF COMMON NICOTINOID ALKALOIDS AND Nacy|INN FORMS IN LEAF EXUDATE OF *Nicotiana* SPECIES AND HYBRIDS^a

Species or hybrids	Anatabine	Anatabine	Nicotine	Normicotine	Acetyl-Formyl Normicotine	Nacy INN	OH-Nacy INN
<i>N. tabacum</i>	1.00	2.11	94.5	0.5	2.02		
<i>N. sylvestris</i>	10.9		21.8		67.2		
<i>N. stocktonii</i>	0.05	0.08	1.83	0.4	22.0	0.88	74.8
<i>N. repanda</i>	0.02		0.01	0.01	26.4	8.7	64.8
<i>N. repanda</i> × MD 609	58.7		15.6		25.6		
<i>N. repanda</i> × MD 872	0.02		0.26		46.2	4.65	48.9
<i>N. repanda</i> × <i>sylvestris</i>			0.14	0.18	18.3	21.2	60.2
<i>N. gossei</i>	2.05	1.12	95.1	1.03	0.75		
<i>N. benthamiana</i>	18.4		80.8	0.79			
C1 × SRI	13.0	1.27	75.2		0.40		
C3 × SRI	14.7		56.5		28.7		
<i>N. stocktonii</i>		C ₁₂ -acyl	C ₁₂ OH-acyl	C ₁₃ acyl	C ₁₃ OH-acyl	C ₁₄ OH-acyl	C ₁₅ OH-acyl
<i>N. repanda</i>		0.1	0.6	0.1	4.8	83.2	10.5
<i>N. repanda</i> × <i>sylvestris</i>		8.6	2.2	3.2	6.3	72.5	7.1
<i>N. repanda</i> × MD 872		12.8	2.9	16.7	17.3	45.2	4.9
		4.2	3.4	4.5	19.5	59.1	8.4

^aPlant leaves were surface extracted by submersion in acetonitrile for 6 sec. The extract was dried under vacuum and then analyzed for the distribution of nicotine alkaloids and Nacy|INN forms.

larval mortality, while the *N. sylvestris* parent imparted no resistance and did not adversely affect larval growth and feeding (Table 2). The hybrid imparted 72% mortality, and the confidence interval indicates that this hybrid will induce a minimum of 36% mortality above that of KY 14 (Table 3). Only 8% of the larvae feeding on this cross fed heavily, and the confidence interval indicates that the hybrid will permit a minimum of 58% fewer larvae feeding heavily than on KY 14 (Table 3). No larvae attained the third instar while feeding on this cross.

This cross produced 11.2 ± 1.0 μg of NacylNN per cm^2 of leaf surface or 140 ± 2.4 $\mu\text{g}/\text{mg}$ exudate. The quality of the nicotinoid alkaloids was very similar to that of the parent *N. repanda*. With respect to *N*-acylnornicotines, there was a distinct shift toward C_{12} and C_{13} forms in the hybrid at the expense of C_{14} and C_{15} forms (Table 4).

Nicotiana repanda X *N. tabacum* (IIA). This hybrid produced only low levels of mortality and had little adverse effect on larval growth and feeding (Table 2). Further, it failed to produce consistent levels of NacylNN. Some of the plants occasionally produced the alkaloid at the lower limits of detection on TLC. GC analysis showed that the long chain (C_{12} - C_{16}) NacylNN forms were absent (Table 4).

Nicotiana repanda X *N. tabacum* (IIB). This cross produced high levels of mortality and adversely affected larval growth and feeding (Table 2). This was comparable to the *N. repanda* X *N. sylvestris* hybrid with 74% mortality, and the confidence interval indicates it will cause an additional minimum 49% mortality above that of KY 14 (Table 3). Furthermore, no larvae fed heavily nor did they attain the third instar while feeding on this hybrid. This hybrid produced an abundant level of NacylNN, with 6.83 μg of NacylNN per cm^2 or 97.5 ± 13.5 μg of NacylNN per mg exudate. As with the *N. repanda* X *N. sylvestris* hybrid, the quality of the nicotinoid alkaloids was very similar to that of the *N. repanda* parent. Also similar was the shift in *N*-acylnornicotines toward the C_{12} and C_{13} forms (Table 4).

Other Repandae Hybrids (III, IV). Most of the hybrids of tobacco with *N. stocktonii* or *N. nesophila* failed to affect larval growth and development differently than *N. tabacum*. One of the crosses (R 25) imparted 33% mortality (10% mortality above that of the KY 14 control) (Tables 2 and 3). However, the percentage of larvae feeding heavily was not significantly different from KY 14. It is interesting to note that the morphology of this hybrid most closely resembles that of *N. tabacum*. TLC analysis showed that over 95% of the leaf exudate alkaloid in the hybrids was nicotine. In some crosses, very low levels of *N*-formyl/*N*-acetyl nornicotine were present. Topical application of 1 mg of crude exudate from this hybrid to each of 10 second-instar larvae produced 20% mortality, confirming the low toxicity of the exudate and a resistance factor below a level detectable by TLC.

N. tabacum X *Nicotiana benthamiana* (VIA and VIB). As demonstrated by Burk and Stewart (1971), *N. benthamiana* produced high levels of mortality (71%) to *M. sexta* larvae (Table 2). The confidence interval indicates that it will induce a minimum 44% additional mortality above KY 14 (Table 3). In addition, only 42% of the larvae that fed on *N. benthamiana* fed heavily, and the confidence interval indicates that the species induces an additional 16% fewer larvae feeding heavily than on KY 14. Only 24% of the larvae that fed on this species attained the third instar (minimum of 55% fewer larvae will reach this growth stage than on KY 14, by confidence interval). None of the *N. benthamiana* hybrids produced levels of mortality near that of the wild parent (Table 2). This analysis is further complicated by the fact that the biochemical basis of resistance in this wild species is not known; hence there is no marker with which to trace the transfer of the resistance into the hybrid. Also, while both retained high nicotine content, only one gained *N*-formyl/*N*-acetylNIN production (Table 4).

Nicotiana gossei X *N. tabacum* (V). *Nicotiana gossei* produced 33% mortality to hornworm larvae. According to the confidence interval, we can expect that this hybrid will cause a minimum of 10% additional mortality above that of KY 14 (Table 3). The percentage of larvae feeding heavily on this species was not significantly different from the KY 14 control. Not surprisingly, the hybrid of this species and *N. tabacum* failed to produce significant levels of mortality and did not adversely affect larval growth and feeding (Table 2).

Hybrid Trichome Morphology. Inasmuch as NacylNIN is produced in leaf trichomes (Zador and Jones, 1986), it was relevant to examine the trichome types in hybrids that did or did not produce the compound. The leaf trichomes of *N. repanda* differ from those of *N. sylvestris* in that they are more stout, less densely placed on the leaf, and are more heavily exudated (Figure 1A,B). The hybrid between these two species expressed trichome characteristics intermediate between those of the parents (Figure 1C). In contrast, the trichome characteristics of the hybrid between *N. repanda* and MD 609 were very similar to *N. tabacum* (Figure 1D,E). This hybrid did not produce NacylNIN, whereas the former hybrid did.

DISCUSSION

In the present study, we have examined NacylNIN production and insect resistance in *Nicotiana* species and hybrids with tobacco. A total of five Repandae hybrid types and two Suaveolentes hybrid types were examined. Two of the Repandae hybrids expressed the *N*-acylnornicotine-based insect resistance, while the other three showed little or no expression of *N*-acylnornicotines.

The interpretation of results of hybridizations using *N. repanda* is expedited by the ease of testing for *N*-acylnornicotine in the leaf exudate. Wide

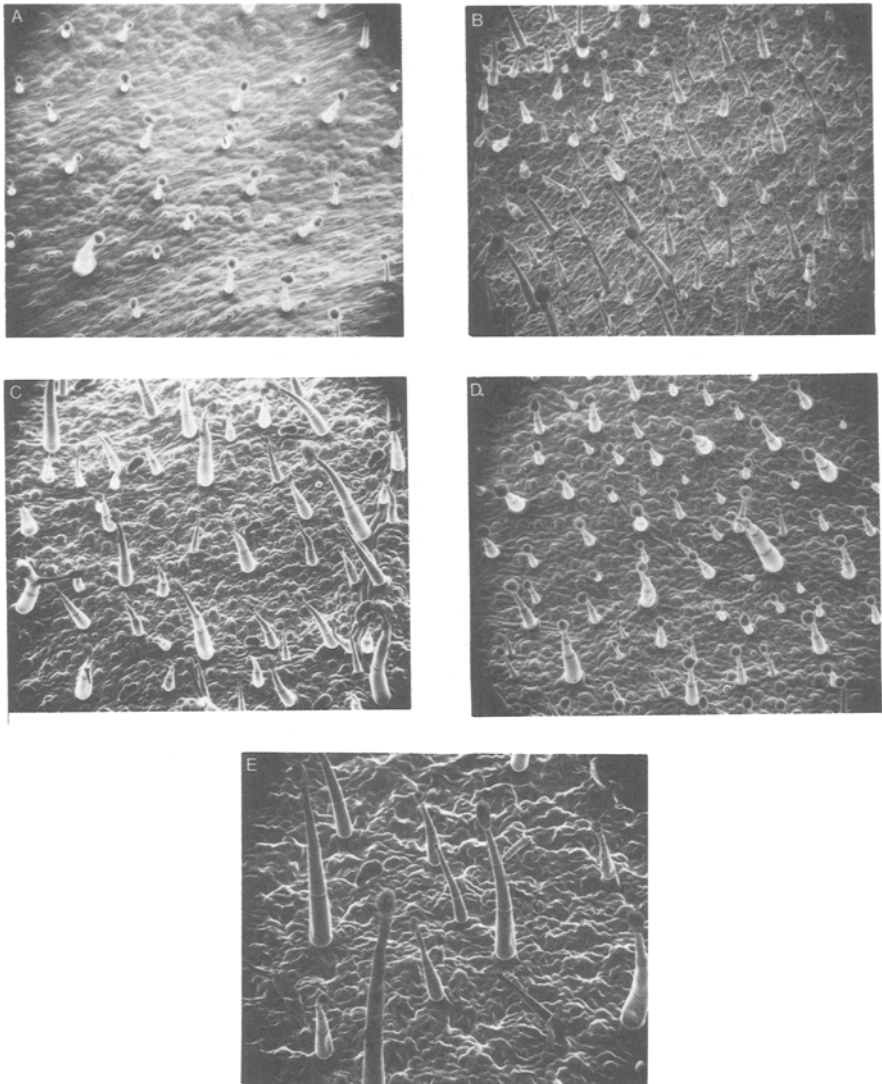


FIG. 1. Scanning EM of trichomes on the leaf surface of various *Nicotiana* spp. and wide species hybrids. (A) *N. sylvestris* (65X), (B) *N. repanda* (65X), (C) *N. sylvestris* X *N. repanda* hybrid (65X), (D) *N. tabacum* (KY 14) (65X), (E) *N. repanda* X *N. tabacum* (135X).

species hybridization between *N. tabacum* and *N. repanda* has long been considered difficult or impossible due to problems of seeding lethality (Iwai et al., 1985; Reed and Collins, 1978; Pittarelli and Stavely, 1975), and previous reports of this hybridization have not been repeated. However, the uniqueness of the *N*-acylnornicotines makes them an unambiguous marker, and there can be no doubt that the hybrids we have examined contain *N. repanda* genetic material. This marker should be useful in rapid confirmation of the success of other or future hybridization efforts using *N. repanda*.

In most previous studies, little correlation was found between the quality of alkaloids in a given *Nicotiana* species and its phylogenetic position (Smith, 1942; Smith and Abashian, 1963; Saitoh et al., 1985). However, it has recently been shown that the *N*-acylnornicotine group of alkaloids is in high concentration in the leaf exudate of all three members of the section Repandae, and it does not occur in such abundance in the exudate of any other *Nicotiana* species (Huesing and Jones, 1987; Severson et al., 1985a,b, 1987).

Both of the hybrids with *N. repanda* found to express *N*-acylnornicotine synthesis have been reported previously to possess disease resistance (Pittarelli and Stavely, 1975; Burk, 1967), and in the bridging species study, the hybrid obtained after the second bridge cross also expressed disease resistance (Burk, 1967). Those results are very interesting in relation to recent data that the *N*-acylnornicotines possess antibacterial activity (Cutler et al., 1986). Perhaps this group of compounds, so toxic to *M. sexta*, will also be found to be the basis, at least in part, for the disease resistance that has made *N. repanda* such appealing material in breeding efforts. The original hybrid obtained through the bridge cross is now being back-crossed to *N. tabacum* (Gwynn, personal communication). It will be interesting, from a genetic and a practical perspective, to monitor expression of the alkaloid and its associated insect resistance in each back-cross generation. Advances in plant tissue culture techniques have now made it possible to obtain novel wide species hybrids, and although there has been interest in this approach with respect to insect resistance (Heinrichs et al., 1985), no positive results have been published. In the present study hybrids between *N. tabacum* and Repandae species produced by fertilized ovule culture (Reed and Collins, 1978) were examined. The hybrid did not express the gene for insect resistance. More research needs to be done on the genetics of the chromosomal material from the Repandae parent which was transferred in the two successful hybrids we examined.

Researchers studying inheritance of alkaloids in wide species hybrids have been exasperated, in some cases, by the apparent complexity of factors governing alkaloid biosynthesis. Smith and Abashian (1963) commented on the need for a simple genetic system to create a base of information upon which studies of more complex systems could be done. The synthesis of *N*-acylnornicotine in Repandae species and hybrids with them appears to offer such a system.

The data reported by the present study (Table 4) using this system provide information relative to inheritance of nicotinoid alkaloids and interactions in the expression of alkaloid biosynthesis. The data suggest the following:

1. Formylation–acetylation is distinct from long-chain *N*-acylation. The evidence for this conclusion is that both *N. repanda* and *N. stocktonii* have formylation–acetylation and *N*-acylation activity, but the hybrid *N. repanda* X MD 609 possesses only formylation–acetylation activity. Also, the hybrid C3 X SRI as well as *N. sylvestris* have formylation–acetylation activity but no *N*-acylation activity. These data also confirm that long-chain *N*-acylation is enzymatic, instead of just an organochemical condensation of free carboxyl groups and pyrrolidine nitrogens. Otherwise, the above-mentioned species and hybrids would not show specificity for formylation/acetylation activity.

2. The formylation–acetylation and *N*-acylation activities are competitive for the same normicotine substrate pool. The evidence for this conclusion is that in *N. sylvestris* formyl–acetylINN comprised 67% of the leaf surface alkaloids, while in its hybrid with *N. repanda* (which produced *N*-acylINN) this percentage dropped to 18%. If the normicotine substrate for formylation–acetylation was inaccessible to the *N*-acyltransferase, formation of long-chain NacylINN would not have occurred in the hybrid at the expense of production of *N*-formyl/*N*-acetylINN. Since acylation occurs in the trichomes (Zador and Jones, 1986), the normicotine that finds its way to the leaf surface as *N*-formyl/*N*-acetylINN does not become formylated–acetylated prior to arrival in the trichomes. That is, formylation–acetylation must occur in the trichomes for it to be competitive with *N*-acylation. On this basis, it would be predicted that formyl–acylINN is in disproportionately low abundance in the leaves of trichomeless mutants. It would also be predicted that at least a part of the formylation–acetylation activity is enzymatic; otherwise the alternative spontaneous organochemical reaction could occur anywhere in the plant (on route to the leaf) uncompetitively with *N*-acylation.

3. Formylation–acetylation activity and anabasine biosynthesis are not linked, and either can appear in hybrids of parents which do not express them. The evidence for these conclusions is the differences in proportional expression of formyl–acylINN and anabasine in C1 X SR1 vs. C3 X SR1. Furthermore, formyl–acetylINN do not occur in the *N. benthamiana* parent of these two hybrids. Also, proportional production of anabasine by the hybrid *N. repanda* X MD 609 was much higher than that of either parent species. Further, the dramatic proportional increase in biosynthesis of formyl–acylINN in C3 X SRI was at the expense of nicotine, rather than anabasine, which is consistent with formylation–acetylation being a terminal, instead of an early, biosynthetic step.

4. The gene for expression of *N*-acyltransferase is probably expressed in a dominant manner. The evidence for this conclusion is that NacylINN was produced in the *N. repanda* X *N. sylvestris* hybrid at a level similar to that of

the *N. repanda* parent. Also, a similar level of expression was observed in the *N. repanda* X *N. tabacum* (MD 872) hybrid. If the inheritance of the allele for *N*-acylnornicotine production follows from a straightforward segregation pattern, it would be anticipated that most plants of these two hybrids would carry one or two alleles. The hybrid with *N. repanda* involving three back-crosses to Md 609 would not be expected to yield, without selection, a large number of plants carrying an allele for *N*-acylnornicotine production, and the plants from this cross were not observed to be strongly resistant, as a group, to *Manduca sexta*. Nornicotine production in *N. tabacum* is substantially the end of the nicotine biosynthesis pathway prior to secretion of nornicotine into the metabolically inert (for nornicotine) leaf exudate. A priori, it would be anticipated that *N*-acylation of nornicotine prior to secretion would be expressed in a dominant manner. Similar reasoning has been used to explain the dominant inheritance of nicotine demethylation to nornicotine (Legg et al., 1969). These data suggest that if the chromosome carrying the *N*-acyltransferase allele is not lost in early generations of a breeding program, then the gene for the enzyme will be fully expressed in the foreign background, given comparable level of substrate.

5. The length of the acyl chain of *N*-acylINN is a manipulatable character. The evidence for this conclusion is that the C₁₄ and C₁₅ forms were predominant in the parent Repandae species, while the C₁₂ and C₁₃ acyl forms were predominant in the hybrids (Table 4). This aspect is very important in view of recent data that the length of the acyl chain directly influences the toxicity of the compound to *M. sexta* larvae (Scarborough and Jones, unpublished data). In a test of C₂-C₁₆ forms, they found that C₁₄ and C₁₆ forms were significantly more toxic than forms with shorter chain length. These data reinforce the point that the fatty acid composition of the background into which the *N*-acyltransferase is placed may permit full expression of the biosynthetic capacity of the enzyme and yet not be proper for production of the forms of NacylINN toxic to *M. sexta*.

The tractability of the genetics of NacylINN production system, the effectiveness of the NacylINN against *M. sexta*, and the ease of monitoring expression of NacylINN biosynthesis also make the *Nicotiana*-*M. sexta* system optimal for testing the concept of genetically engineering plants for nonprotein insect resistance factors. There has been increasing interest in the application of plant molecular biology for the transfer of insect resistance genes (Brill, 1985), but current efforts have focused on genes for which the protein product is, in itself, the toxic agent (Crawford, 1986). The focus on toxic proteins is due to the perception that transfer of genes for production of nonprotein factors will entail the transfer of a number of genes belonging to a biosynthetic pathway (Foard et al., 1983). It has been suggested, however, that many of the plant resistance compounds occurring in wild plant species are only one or a few enzymatic steps away from a substrate already occurring in the cultivated plant. Therefore,

transfer of the gene(s) for the terminal enzymatic modification of that substrate is a logistically practical approach (Jones et al., 1987). The *M. sexta*-*N. tabacum*-*N*-acetylornicotine interaction appears well suited as a model system for testing concepts in the molecular biology of plant resistance to insects and for comparison of such an approach with conventional, bridging species and plant tissue culture methods.

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HOST EGG KAIROMONES ESSENTIAL FOR EGG-LARVAL PARASITOID, *Ascogaster reticulatus* WATANABE (HYMENOPTERA: BRACONIDAE)
II. Identification of Internal Kairomone

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Abstract—Several components of an internal kairomone were identified inside eggs of the host, *Adoxophyes* sp. (Lepidoptera: Tortricidae), that releases egg deposition of the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). Pupal hemolymph with the same activity as an internal host egg kairomone was used as a convenient test sample. Heat-treated pupal hemolymph was chromatographed on a Sephadex G-25 column. Each fraction was bioassayed and reacted with ninhydrin. The active fractions were ninhydrin-positive. Each fraction was placed onto an amino acid analyzer, which showed that the amino acids were most abundant in active fractions. Among 22 amino acids, alanine, arginine, glycine, histidine, isoleucine, leucine, methionine, proline, serine, tryptophan, and valine were active. The mixture of these active amino acids was as active as the egg-mass homogenate at the same ratio and concentration, suggesting that the most important component as the kairomone in a host egg is the mixture of several amino acids.

Key Words—Kairomone, oviposition stimulant, amino acid, parasitoid, *Ascogaster reticulatus*, Hymenoptera, Braconidae, *Adoxophyes* sp., Lepidoptera, Tortricidae.

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INTRODUCTION

For the artificial rearing of insect parasitoids, oviposition by adults into artificial substrates is often necessary (Thompson, 1986). Among factors involved in the process of host acceptance, semiochemicals have been found to play important roles (Arthur, 1981). Different chemical cues may lead the parasitoids to oviposit in this process, like most of the parasitoids locating their hosts with a series of cues (Vinson, 1977).

Both external and internal kairomones were found to be essential for oviposition of the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae) by using Parafilm-based artificial eggs (Kainoh and Tatsuki, 1988). External kairomone, needed for host location and acceptance was extracted with 70% ethanol from egg masses of the host, *Adoxophyes* sp. (Lepidoptera: Tortricidae) (Kainoh et al., 1982). The internal kairomone, needed for oviposition, was extracted with water from host eggs. Interestingly, the same activity was found in the extracts not only from host eggs but also from host larvae and pupae, and even larvae of other lepidopterous and coleopterous insects (Kainoh and Tatsuki, 1988). These results indicate that the internal kairomone is a universal substance(s) and is common in insect hemolymph.

There are just a few cases in which the oviposition stimulant inside the host was identified in parasitoids, i.e., *Itopectis conquisitor* (Say) (Ichneumonidae) (Arthur et al., 1969, 1972; Hegdekar and Arthur, 1973) and *Trichogramma pretiosum* Riley (Trichogrammatidae) (Nettles et al., 1982, 1983).

In the present paper, the internal kairomone was identified by chemical analysis combined with a bioassay using the Parafilm-based artificial egg, and possible role of the chemical is discussed in relation to host specificity.

METHODS AND MATERIALS

Insects. Female parasitoids used in the present experiments were obtained from the stock culture in the University of Tsukuba. The rearing and maintenance of the parasitoids were based on the method by Kainoh and Tamaki (1982) and Kainoh (1986). Emerged adults were sexed, and the females were put into containers (15 cm diam. × 12 cm high) at 20 females per container. Three-day-old virgin females were used for bioassay.

Bioassay. An artificial egg for the bioassay was described by Kainoh and Tatsuki (1988). It consists of two sheets of Parafilm and a test solution (30 μ l) between them. The external kairomone [0.06 egg mass equivalent (EME)] was applied on the upper Parafilm to elicit the oviposition behavior. A 3-day-old female parasitoid was allowed to oviposit on this artificial egg for 30 min, and

the number of parasitoid eggs laid in the test solution was counted under a dark-field stereomicroscope.

Collection of Sample. Since host egg masses are too small to collect hemolymph, and the supernatant of the homogenized host pupae was as active as that of egg masses (Kainoh and Tatsuki, 1988), host pupal hemolymph was used for the sample. This was based on the assumption that the active chemical(s) in the host egg must also be present in the pupae. The frozen pupae were cut with a blade and put into a centrifugal tube (1.5 cm diam. \times 7.5 cm long) with a horizontal mesh filter in the middle of the tube. It was centrifuged at 7000g for 30 min, and the greenish supernatant (hereafter designated as crude hemolymph) was pipetted out and kept in a freezer (-20°C) until use.

Heat Stability. The pupal hemolymph (5 ml) in a test tube was heated in a water bath (100°C) for 10 min, and it was centrifuged at 7000g for 30 min. The supernatant (2.4 ml) was diluted stepwise with distilled water from two to 64 times. The activity of each sample of heated hemolymph was compared with that of diluted sample of the crude hemolymph.

Ion-Exchange Column Chromatography. To analyze the pupal hemolymph, a cation-exchange resin (Amberlite IR-120) column ($15 \times 90\text{mm}$) was used. After 2 ml of the crude pupal hemolymph was loaded on the column, 100 ml of distilled water was passed through the column and the washing was collected. Then the sample was eluted twice each with 100 ml 4 N HCl. The eluates were concentrated to dryness under reduced pressure and taken up in 10 ml distilled water. These water and 4 N HCl eluates were neutralized with 0.1 N NaOH solution before bioassay. Then 2 ml crude hemolymph was diluted with 100 ml distilled water and treated the same way as the 4 N HCl eluates. The diluted sample (10 ml) was neutralized and bioassayed as the control.

Sephadex G-25 Column Chromatography. For further analysis of the pupal hemolymph, column chromatography with Sephadex G-25 (fine) was performed. Since the heated pupal hemolymph was active in the heat stability experiment, it was used as a sample. A sample of 2 ml heated pupal hemolymph, which was treated as in the heat stability experiment, was loaded on the column ($15 \times 970\text{ mm}$) and eluted with 250 ml distilled water. The flow rate was adjusted to ca. 15 ml/hr. Each fraction of 5 ml was collected, and the UV absorbance was monitored at 280 nm. The first bioassay was performed for every other fraction ($N = 3/\text{sample}$) and the second for every fraction from 27 to 33 ($N = 3-6/\text{sample}$).

Amino Acid Analysis. Total amount of amino acids of each fraction from Sephadex G-25 column chromatography was estimated by ninhydrin procedure. Fractions 25-38, including active fractions, were chromatographed using an amino acid analyzer (Hitachi, model 835).

To determine the amino acid composition of host materials, 50 *Adoxophyes*

sp. egg masses (ca. 0.2 g) were homogenized with 1 ml of 2% sulfosalicylic acid (SSA) solution in a glass mortar with a pestle to remove protein and centrifuged at 7000g for 10 min. The supernatant was diluted 10 times with 0.02 N HCl. Next the crude pupal hemolymph (100 μ l) of *Adoxophyes* sp. was mixed with 200 μ l of 2% SSA solution and centrifuged at 7000g for 10 min. The supernatant was diluted 10 times with 0.02 N HCl. These samples were loaded on an amino acid analyzer after filtration through a membrane (pore size: 0.45 μ m).

Activity of Amino Acids. In order to determine the active amino acids, ovipositional activity of the following authentic L-form amino acids was tested; α -alanine (α -Ala), β -alanine (β -Ala), arginine (Arg), aspartic acid (Asp), asparagine (Asn), cysteine (Cys), cystine [(Cys)₂], glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val). Each amino acid was dissolved in distilled water at the concentration of 0.1 M and 0.01 M, but slightly soluble amino acids were only at 0.01 M or saturated solution. The pH of each solution was adjusted to 7 with 0.01–0.1 N HCl or 0.01–0.1 N NaOH. Their ovipositional activity was compared to those without pH adjustment. Some of the active amino acids (histidine, methionine, proline, and serine), at various concentrations and without pH adjustment, were prepared and bioassayed. In the case of histidine, saturated solutions were prepared at 0.27 M.

Activity of Amino Acid Mixture. The two mixtures of authentic amino acids were prepared at the same concentrations of amino acids as in the host egg-mass homogenate and the pupal hemolymph. Based on the amino acid composition (Table 1) and active amino acids (Table 2), one mixture of 11 active amino acids (Ser, Pro, Gly, α -Ala, Val, Met, Ile, Leu, Trp, His, Arg) and another mixture of 20, including the 11 active and nine inactive or slightly active ones, were prepared in the concentrations corresponding to those of the egg-mass homogenate and the pupal hemolymph. These mixtures were bioassayed with and without pH adjustment by 0.1 N HCl or 0.1 N NaOH.

To compare the activity of above mixtures with that of host egg-mass homogenate, 50 egg masses (ca. 0.2 g) were homogenized with 1 ml distilled water and centrifuged at 7000g for 10 min for the supernatant to be bioassayed ($N = 35$).

RESULTS

Heat Stability. There was no ovipositional activity in the original pupal hemolymph and those diluted twice and four times. The activity was found at eight and 16 times dilutions, with the highest activity at 16 times ($\bar{X} = 47.4$).

In the case of heated hemolymph, activity emerged at four, eight, and 16 times dilution with the highest activity at 8 times ($\bar{X} = 52.2$).

Ion-Exchange Column Chromatography. There was no ovipositional activity in the eluate of distilled water from ion-exchange column chromatography, but the activity emerged from the first eluate of 4 N HCl ($\bar{X} = 30.2$). The second eluate of 4 N HCl was slightly active ($\bar{X} = 2.2$). The activity of the first 4 N HCl eluate was not significantly different ($P < 0.05$) from the control ($\bar{X} = 23.0$), but that of the second eluate was significantly lower ($P < 0.05$) than the control as determined by Duncan's multiple range test.

Sephadex G-25 Column Chromatography. The activity was found in fractions 29–31 from Sephadex G-25 column chromatography (Figure 1). The peak of the ovipositional activity was in fraction 30 with 31.7 eggs in average. The ninhydrin coloration peak was found in fractions 28–30.

Amino Acid Analysis. Among the fractions from Sephadex G-25 column, most of the peaks of the amino acids were in fractions 29–31 (Table 1), and the total concentration of 22 amino acids was highest in fraction 30 (29: 4.7, 30: 7.2, 31: 4.7 mM), which coincided with the activity peak.

In the egg-mass homogenate, glutamine, glutamic acid, arginine, and histidine were abundant; however, α -alanine and proline were most abundant in the pupal hemolymph (Table 1).

Activity of Amino Acids. There were 11 active amino acids, i.e., α -alanine, arginine, glycine, histidine, isoleucine, leucine, methionine, proline, serine,

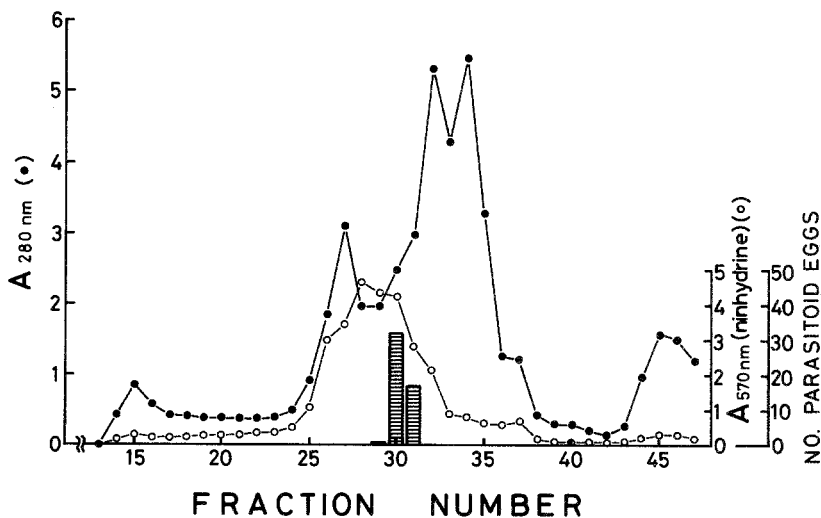


FIG. 1. Sephadex G-25 column chromatography. Sample: 2 ml of heated pupal hemolymph of *Adoxophyes* sp. Column: 15 × 970 mm. Flow rate: 15 ml/hr. Striped bars show the ovipositional activity ($N = 6$ /sample).

TABLE 1. AMINO ACID COMPOSITION (mM) OF FRACTIONS FROM SEPHADEX G-25 COLUMN CHROMATOGRAPHY (1.5 × 97 cm) COMPARED WITH PUPAL HEMOLYMPH AND EGG-MASS HOMOGENATE OF *Adoxophyes* sp.

Amino acids	Fraction number					Pupal hemolymph ^a	Egg-mass homogenate ^a
	28	29	30	31	32		
Asp				t ^b	t	0.04	0.39
Thr	0.03	0.17	<u>0.34</u> ^c	0.21	0.04	2.02	0.38
Ser	0.01	0.08	<u>0.44</u>	<u>0.47</u>	0.08	3.49	0.62
Asn	—	—	0.12	<u>0.22</u>	0.07	1.47	0.74
Glu	0.39	0.28	0.15	<u>0.03</u>	0.02	6.50	1.99
Gln	0.01	0.07	0.49	<u>0.63</u>	0.10	5.18	2.83
Pro	0.36	1.25	1.15	<u>0.41</u>	0.01	8.90	0.81
Gly	0.01	0.08	0.60	<u>0.72</u>	0.18	5.32	0.60
α-Ala	0.21	1.08	<u>1.57</u>	<u>0.75</u>	0.09	9.20	0.39
Val	0.11	<u>0.38</u>	<u>0.36</u>	0.14	0.02	2.93	0.70
Cys + (Cys) ²		t	0.01	<u>0.05</u>	t	0.56	0.01
Met	0.10	0.14	0.07			1.14	0.30
Ile	0.04	<u>0.18</u>	<u>0.26</u>	0.14	0.02	2.17	0.27
Leu	0.04	0.30	<u>0.52</u>	0.30	0.04	3.46	0.47
Tyr			t	t	t	4.82	0.81
Phe					0.01	1.26	0.24
β-Ala	0.01	0.05	<u>0.06</u>	0.03	t	0.55	0.08
Trp						1.00	0.04
Lys	0.38	0.33	0.03	t	t	3.89	0.75
His	t	t	0.07	0.16	0.16	3.90	1.38
Arg	0.01	0.42	<u>0.84</u>	0.24	0.05	5.98	1.61
Ovipositional activity (avg. No. eggs)	0	0.8	31.7	16.8	0	0	56.9

^a Average values of three replications.

^b t = trace.

^c A peak value of each amino acid is underlined.

tryptophan, and valine (Table 2). Among these, 0.1 M solutions of histidine and methionine were most active both at pH 7 and without pH adjustment. Histidine was still active even at 0.01 M. α-Alanine, leucine, and valine were less active (0.1 M). The activity of glycine and serine increased with pH adjustment (pH 7), but in isoleucine and proline, it decreased with pH adjustment. Only slight activity was found in the 0.1 M solution of β-alanine (pH 7), 0.1 M cysteine (pH 7), 0.1 M lysine (pH 7), 0.1 M and 0.01 M phenylalanine (pH 7), and in the saturated solution of cystine (0.5 mM) (pH 5.9). The 0.01 M arginine was active after pH adjustment, although the 0.1 M solution was not so active even after pH adjustment.

In the dose-response relationship of methionine, the highest activities were obtained at 0.05 M ($\bar{X} = 90.7$), 0.1 M ($\bar{X} = 92.7$), and 0.2 M ($\bar{X} = 93.7$) (Figure 2). The activity of histidine increased to the maximum ($\bar{X} = 89.4$) as

TABLE 2. OVIPOSITIONAL ACTIVITY OF VARIOUS AMINO ACIDS FOR *A. reticulatus*

Amino acid ^a	Ovipositional activity (avg. No. eggs)							
	Concentration							
	0.1 M				0.01 M			
No.	pH not adjusted	(pH)	pH 7 ^b	No.	pH not adjusted	(pH)	pH 7 ^b	
α -Ala*	6	79.7	(6.1)	45.7	6	1.2	(5.7)	2.2
β -Ala	6	0	(6.8)	9.7	3	0	(6.6)	0
Arg*	6	0	(10.7)	6.8	6	0	(10.0)	55.0
Asp					6	0	(3.1)	0
Asn	6	0	(4.3)	0	3	0	(4.9)	0
Cys	6	0	(5.2)	1.7	3	0	(5.6)	0
(Cys) ₂ (0.5 mM) ^c					6	0.2	(5.9)	0
Glu					6	0	(3.4)	0
Gln	6	0	(5.3)	0	3	0	(5.7)	0
Gly*	6	0	(6.1)	29.2	3	0	(6.0)	0
His*	5	89.4	(7.6)	82.8	5	16.8	(7.7)	12.6
Ile*	6	28.5	(6.6)	6.3	3	0	(6.7)	0
Leu*	6	61.0	(6.9)	64.2	3	0	(6.5)	0
Lys	6	0	(9.4)	0.2	3	0	(9.4)	0
Met*	6	92.7	(5.9)	64.2	3	0	(6.1)	0
Phe	6	0	(5.8)	9.7	6	0	(7.2)	0.5
Pro*	6	33.5	(6.2)	18.8	3	0	(6.1)	0
Ser*	6	8.5	(5.3)	53.3	6	0	(5.8)	0
Thr	6	0	(6.4)	0	3	0	(6.6)	0
Trp*					6	27.3	(7.0)	0
Tyr (25 mM) ^c					6	0	(6.9)	0
Val*	6	32.8	(6.4)	56.0	3	0	(6.2)	0

^aAll amino acids used were L form. Those with asterisks (*) were regarded as active amino acids.

^bThe pH was adjusted to 7 with NaOH or HCl solution.

^cSaturated solution was prepared.

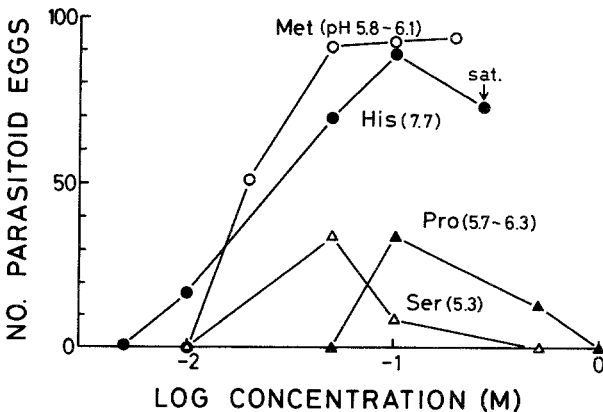


FIG. 2. Dose-response curves of some amino acids ($N = 5-6$ /sample).

TABLE 3. ACTIVITY OF AMINO ACID MIXTURE SIMULATING THE HOST EGG-MASS HOMOGENATE FOR OVIPOSITION OF *A. reticulatus*

No. of amino acids mixed ^a	pH ^b	No. of tests	Ovipositional activity (avg. No. eggs) ^c
11	8.7	15	6.5 ± 4.4 c
	7.0	15	62.1 ± 6.9 b
20	6.5	15	92.3 ± 4.0 a
	7.0	15	73.7 ± 9.7 b
Egg-mass homogenate ^d	6.9	35	56.9 ± 4.7 b

^aMixtures of 11 active amino acids and 20 amino acids including inactive ones [except Cys and (Cys)₂] were tested. The ratio and concentration were based on Table 1.

^bThe pH was adjusted to 7 with 0.1 N HCl or 0.1 N NaOH.

^cValues are $\bar{X} \pm SE$ ($N = 10$). Means followed by the same letters are not significantly different (5% level) as determined by Duncan's multiple-range test.

^dFifty host egg masses were homogenized with 1 ml of distilled water ($N = 35$).

the concentration increased to 0.1 M, then slightly decreased ($\bar{X} = 73.2$) in the saturated solution (0.27 M). In the case of histidine and proline, the maximum activity was obtained at 0.1 M, and it decreased as the concentration increased; however, serine had the highest activity ($\bar{X} = 34.3$) at 0.05 M. In the highest concentrations of proline (1 M) and serine (0.5 M), the activity was only slight or null.

Activity of Amino Acid Mixture. Activity of the mixtures of both 11 and 20 amino acids in the concentration simulating the egg-mass homogenate was not significantly different at pH 7 from that of real egg-mass homogenate ($\bar{X} = 56.9$) (Table 3).

In the case of amino acid mixtures simulating the pupal hemolymph (Table 4), all the mixtures were active, although the pupal hemolymph was inactive. There was no significant difference of activity between the 11 and 20 amino acid mixtures at pH 7.

DISCUSSION

An internal kairomone of *A. reticulatus* was identified as amino acids, which explained the nonspecific ovipositional response to the hemolymphs of several insects of different orders shown by Kainoh and Tatsuki (1988).

The ovipositional activity of diluted pupal hemolymph shows the optimum

TABLE 4. ACTIVITY OF AMINO ACID MIXTURE SIMULATING HOST PUPAL HEMOLYMPH FOR OVIPOSITION OF *A. reticulatus*^a

No. of amino acids mixed	pH	No. of tests	Ovipositional activity (avg. No. eggs)
11	8.6	15	61.8 ± 11.8 b
	7.0	15	89.1 ± 7.6 a
20	7.7	15	105.5 ± 6.2 a
	7.0	15	95.1 ± 6.6 a
Pupal hemolymph	6.4	10	0 c

^aSee Table 3 footnotes

concentration for oviposition in both crude and heated hemolymph. These data suggest that the active material(s) in the pupal hemolymph is inactive in higher concentrations, otherwise masking or inhibitory substance(s) are included in the pupal hemolymph. The different activity peaks of the two samples may be due to some effect of the material(s) in crude pupal hemolymph.

Ion-exchange column chromatography shows that the active material(s) is positively charged. Sephadex G-25 column chromatography (Figure 1) and amino acid analysis of each fraction from the column (Table 1) show that the activity of pupal hemolymph is due to amino acids. Of 22 amino acids (Table 2), 11 are active at pH 7 and/or with no pH adjustment. The pH of some of these amino acid solutions does not seem to critically affect activity.

Dose-response curves with some amino acids (Figure 2) show that the higher the concentration above 0.1 M, the lower the activity, except methionine, which may be due to the inhibitory effect of higher amino acid concentration. This result cannot explain the inactivity of crude pupal hemolymph, since the mixture of authentic amino acids in a concentration simulating the pupal hemolymph is active (Table 4). Some material(s) in the crude pupal hemolymph may mask the activity of the amino acids. Similar dose-response curves of amino acids were shown by Arthur et al. (1972), in which each active amino acid has an optimum concentration for oviposition of *I. conquisitor*.

The activity of mixtures of 11 and 20 amino acids (pH 7) was not significantly different from that of the egg-mass homogenate at the same ratio and concentration (Table 3). This shows that the main components of the internal kairomone can be the mixture of amino acids. The mixture of 11 active amino

acids (total concn: 7.2 mM) simulating the egg-mass homogenate was active ($\bar{X} = 62.1$) at pH 7 (Table 3), although the individual amino acid was not so active in 10 mM ($\bar{X} = 8.8$, range:0–55.0, $N = 11$) at pH 7 (Table 2). This shows the synergistic effect of the active amino acids.

There are some parasitoid species in which several amino acids have been identified as internal kairomone from the host. In a pupal parasitoid, *I. conquisitor*, the mixture of serine (0.5 M), arginine (0.05 M), leucine (0.065 M), and magnesium chloride (0.025 M) was more effective than the host hemolymph in inducing oviposition (Arthur et al., 1972). Also, in an egg parasitoid, *Trichogramma dendrolimi* Matsumura, leucine, phenylalanine, isoleucine, and histidine were found to be active oviposition stimulants by using artificial eggs (Wu and Qin, 1982). Judging from these cases including *A. reticulatus*, amino acids seem to be common as an internal kairomone for inducing oviposition of endoparasitoids.

Glycine and serine were identified as stimulants for antennal drumming and ovipositor insertion from the host egg-mass hair (scales) in *Tetrastichus schoenobii* Ferriere (Eulophidae) (Du et al., 1982). In *Apanteles cypris* Nixon (Braconidae), trehalose and eight amino acids (leucine, phenylalanine, aspartic acid, serine, cystine, alanine, valine, and threonine) were identified as searching stimulants from the host larval frass (Hu and Chen, 1987). In these species, other specific substance(s) may act as another kairomone to determine their host specificity. In *A. reticulatus*, a specific substance is the external kairomone (Kainoh et al., 1982), which may be the critical factor to determine the host specificity of this species (Kawakami and Kainoh, 1986).

The total amino acid concentration of pupal hemolymph (74 mM) (Table 1) seemed to be higher than the egg hemolymph, considering the total free amino acid concentration (ca. 35–55 mM) of eggs of other lepidopterous species, i.e., *Philosamia ricini*, *Antherae pernyi*, and *Corcyra cephalonica* (Xie et al., 1982). The low concentration of free amino acids in the eggs may be critical for the development of parasitoid larvae, especially for egg parasitoids.

An artificial egg used for the present bioassay can be used to collect parasitoid eggs effectively, which will make it possible to develop an artificial rearing method with artificial media. In the case of *T. pretiosum*, oviposition and in vitro rearing systems are now being developed with artificial eggs (Xie et al., 1986). In larval or egg-larval parasitoids, the method will be more complex and needs intensive research; however, this direction of research will give us some informative cues to find new techniques for biological control.

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METAL COMPOSITION AS A NATURAL MARKER IN ANTHOMYIID FLY *Delia radicum* (L.)

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Abstract—Graphite furnace and flame atomic absorption spectroscopy were used to make quantitative determinations of a range of metallic cation contents of wet-ashed female individuals of *Delia radicum* (L.) from a laboratory culture reared under controlled conditions and freeze-dried upon emergence. Analyses were done for seven elements: Fe, Zn, Cu, Pb, Al, Mg, and K. The quantities of K and Mg present were positively related to the dry weight of individual flies, while the others showed an exponential decrease in concentration with increasing fly weight. This difference is attributed to the different roles of surface adsorption of metals with higher oxidation states and the high absorption efficiencies of those existing as oxidation states 1 and 2. The weight of the insect is therefore a major factor in determining the individual's chemoprint. The implications of this observation for other studies are discussed.

Key Words—Graphite furnace, heavy metals, chemoprints, *Delia radicum*, Diptera, Anthomyiidae, surface adsorption, oxidation states, metal cations.

INTRODUCTION

Dempster et al. (1986) described how the naturally occurring chemical composition of insects may act as a population marker for discriminating between populations under three assumptions: (1) a pattern of elements builds up in the individual insect during its growth and persists into the imago; (2) this pattern is not destroyed by adult feeding or aging; and (3) the pattern of elements is

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characteristic of the locality in which the individual passed through its life cycle, so that the variation between populations outweighs the variation within populations. This technique is most commonly referred to as chemoprinting and appears to have application in studies of insect dispersal (McLean et al., 1979; Turner and Bowden, 1983; Bowden et al., 1984; Dempster et al., 1986).

A diversity of analytical approaches has been used based upon X-rays, and the validity of some of the results obtained has been questioned. Bowden et al. (1979) noted that the relative methods based upon X-ray energy-dispersive spectroscopy (XES) used in the earliest attempts at chemoprinting (McLean and Bennett, 1978; Turnock et al., 1979) had disadvantages compared with the quantitative analysis of specific elements by X-ray fluorescence microanalysis (XRF). The question of analytical sensitivity for these methods is discussed at length by Bowden et al. (1985).

As a consequence of the uncertain validity of X-ray methods, we felt that it would be advantageous to develop a reliable quantitative technique that was not based upon X-rays. Furthermore, we felt that the different selections of elements used for discrimination even within single species (Bowden et al., 1984, 1985) demanded a better understanding of how the elements selected for chemoprinting are actually accumulated in the insect. One special requirement of chemoprinting is that there should be a characteristic nature to any pattern for a given locality or population (Dempster et al., 1986).

Smock (1983) and Darlington et al. (1986) drew attention to the processes of absorption and surface adsorption in determining the concentration of elements in aquatic insect tissue. With these studies in mind, we investigated the use of atomic adsorption spectroscopy (AAS) for analyzing elemental composition of individual adult cabbage root flies [*Delia radicum* (L.); (Diptera; Anthomyiidae)] from cultures maintained in the laboratory as a preliminary to using chemoprinting to study dispersal of this species in the field. In particular, any surface area-to-volume effects were examined to ascertain their potential influence upon discrimination between different sources of insects.

METHODS AND MATERIALS

Rearing of Insects. We used a culture of *D. radicum* maintained at Plymouth Polytechnic for five years originating from locally captured wild flies. The controlled conditions were $20 \pm 1.5^\circ$ C, 16 hr light, and $60 \pm 5\%$ relative humidity, and the culturing technique was similar to that in Finch and Coaker (1969).

Eggs from a culture cage were inoculated onto a single swede (*Brassica napus* L.), and the pupae produced were subsequently isolated. On emergence, the adult flies were frozen and freeze-dried to constant weight.

Preparation and Analysis of Insects. The dried female flies were weighed on a Cahn Automatic Electro-balance 29 (Cahn Instruments, Inc., Cerritos, California), to 0.1 μg . Each fly was placed singly in a numbered vial. Boyden (1974) emphasized the advisability of using as wide a range of specimen weights as possible in studies to evaluate the effect of weight on metal concentrations. Therefore, we took 11 representatives from the range 2–3 mg, nine from the range 3–4 mg, six from 4–5 mg, three from 5–6 mg, and one above 6 mg, the only one of this weight available.

All glassware was acid washed, soaked for 48 hr in 2 M hydrochloric acid, 48 hr in nitric acid, rinsed three times in deionized distilled water, and then dried (Rowland, 1982). Each fly was placed in a dry digestion tube and 1 cm^3 of concentrated Aristar grade nitric acid (BDH Chemicals, Poole, Dorset, U.K.) added. Digestion was then undertaken on a programmable digestion system (Tecator Ltd, Bristol, U.K.). Upon completion, the digests were diluted to 50 cm^3 with deionized distilled water.

Several elements were measured in the samples by atomic absorption spectroscopy, using a Varian AA.975 series atomic absorption spectrometer (Varian Tectron Pty. Ltd, Springvale, Australia), either by flame AAS or by electrothermal atomization with a graphite tube atomizer (details in Ebdon, 1982, McKenzie, 1982).

Analytical reliability was checked by taking a bulk sample of a further 25 female flies with dry weights in the range of 3.3–4.4 mg (total weight of 99.6262 mg) and digesting them in 5 cm^3 of concentrated Aristar grade nitric acid, made back up to 5 cm^3 after digestion with deionized distilled water, and analyzed by flame AAS.

Precision of the method was tested by using 10 replicates of a standard solution for each calibration range analyzed as part of each batch run on the instrument.

RESULTS

Seven of the elements occurred in quantities suitable for measurement by these methods (Ebdon, 1982). The analyses of flies, the bulk sample, and the standard replicates are summarized in Table 1. The results for the bulk sample lie within the range for individual flies and are in accord with measurements of metal concentrations made in other species (Levy and Cromroy, 1973; Cohen et al., 1985).

Table 2 and Figure 1 present the regression analyses for \log_{10} concentration of each metallic cation against \log_{10} dry weight of the fly obtained in this study. There was a steep decline of concentration with increase in fly weight for Al, Pb, Fe, Cu, and Zn, and a shallower decline for K and Mg. A representative

TABLE 1. SUMMARY OF ANALYSES FOR INDIVIDUAL FLIES, BULK SAMPLE, AND STANDARDS BY AAS

Element	Individual mean concentration ($\mu\text{g/g}$)	Limits of range ($\mu\text{g/g}$)	Bulk sample concentration ($\mu\text{g/g}$)	Mean absorbance of standard solution ^a
Aluminium	701.72	269.83-1716.06	70.63 ^b	0.253
Lead	40.32	8.32-127.71	12.60	0.292
Copper	48.28	27.62-100.49	7.53 ^b	0.300
Zinc	10.29	25.30-91.00	95.60	0.169
Iron	57.74	15.47-108.58	85.80	0.388
Potassium	10146.52	7959.46-12500.43	11878.00	0.248
Magnesium	945.94	736.73-1274.20	1158.00	0.230

^aSE for standard solutions all ± 0.001 , except lead ± 0.002 .

^bAbsorbances obtained by flame AAS for the bulk sample for this element were too close to the detection limit to be reliable.

plot of the former pattern is demonstrated in Figure 2 for Pb, showing the considerable change in concentration of the metal over a very limited range of fly weights, at the smallest weights of flies available, validating the selection of the representative weights of individuals used in this study. The latter pattern is characterized by the total quantity of metal contained in the individual, being directly proportional to the dry weight. This relationship is shown for K in Figure 3.

TABLE 2. MODEL 1 REGRESSION CONSTANTS AND CORRELATION COEFFICIENTS FOR EQUATION $Y = a + bX^a$

Element	Regression constant		Correlation coefficient (r)
	a	b	
Aluminium	3.45	-1.18	-0.70***
Lead	2.22	-1.29	-0.55**
Copper	2.00	-0.63	-0.47*
Zinc	1.99	-0.54	-0.48*
Iron	2.25	-0.95	-0.58**
Potassium	4.14	-0.26	-0.62***
Magnesium	3.12	-0.27	-0.51**

^aWhere $Y = \log_{10}$ organism metal concentration ($\mu\text{g/g}$ dry weight) and $X = \log_{10}$ organism dry weight (mg) for *Delia radicum* females. $N = 30$. Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

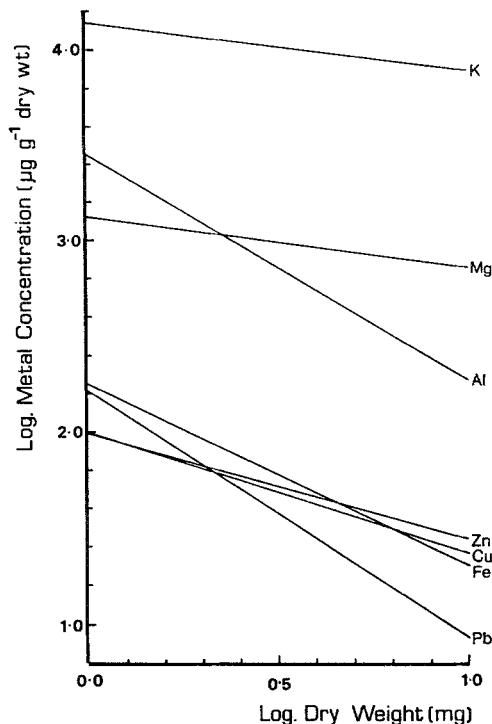


FIG. 1. Relationship of metal concentration and dry weight in female cabbage root fly.

DISCUSSION

Bowden et al. (1984) have pointed out that the use of a method of analysis that allows identification of elements, rather than the relative parameters described as "regions of interest" produced by earlier studies (e.g., McLean and Bennett, 1978; McLean et al., 1979; Turnocket et al., 1979), has the advantage of enabling us to specify unequivocally the elements that make up the chemoprints. For those metallic cations that do not occur in quantities within the analytical range of flame AAS, graphite furnace AAS represents a very satisfactory analytical technique. It is capable of giving measurements of enough elements to provide an adequate chemoprint for individual cabbage root flies.

This study of a laboratory population of *D. radicum* has confirmed observations made in studies of metal uptake by aquatic insects and reviewed by Smock (1983). Furthermore, his identification of two types of general relationships between metal concentration and weight were also confirmed.

Metals that show no disproportionate effect of insect weight upon metal

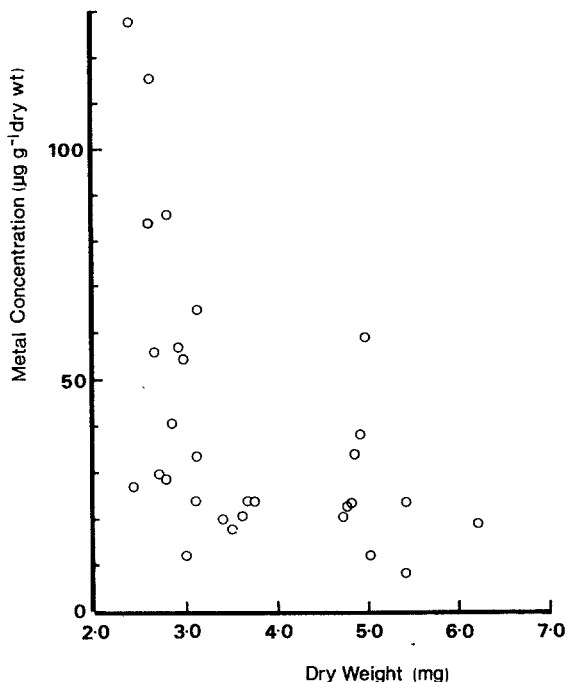


FIG. 2. Relationship between dry weight (mg) and lead concentration ($\mu\text{g/g}$) in individual female cabbage root fly.

concentration, such as K, Mn, and Na, were identified by Smock (1983) as type 1 in his study of *Stenonema modestum* (Banks) and *Stenacron interpunctatum* (Say) (Ephemeroptera; Heptageniidae). We did not measure Na, and Mn did not occur in measurable quantities, but we would include Mg as type 1 since it shows the characteristic relationship of quantity of metal against dry weight exemplified in Figure 3. This relationship occurs where absorption, rather than adsorption, is the more important factor determining metal concentration, since both the number of potential binding sites (e.g., tissues, fluids, enzymes, etc.) and body weight increase simultaneously. Metals of this type tend to have high absorption efficiencies and to exist in low oxidation states and simple ionic forms, which favor absorption as the major mode of accumulation.

Metals of the second type (type 2) show an exponential decrease in concentration with increasing body size, which is linked to the absence of a simple relationship between the quantity of metal present and the individual's dry weight. Smock (1983) identified four type 2 metals, namely, Co, Cr, Fe, and Sc, and linked this effect to the surface adsorption method of accumulation

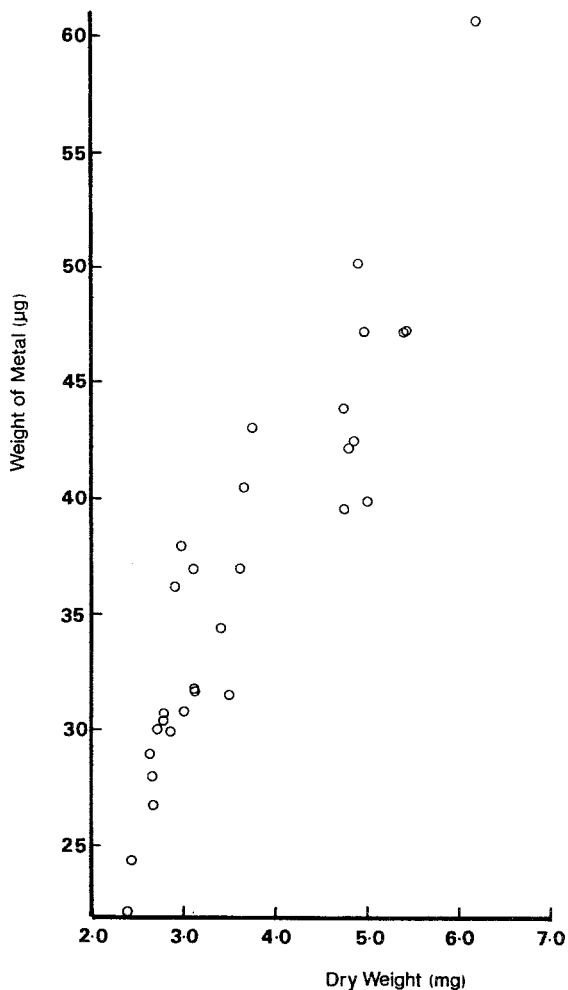


FIG. 3. Relationship between dry weight (mg) and amount of potassium (μg) in individual female cabbage root fly.

typical of elements with higher oxidation states and existing in complex ionic forms. We were unable to measure the low concentrations of Co or Cr in *D. radicum* by AAS, but Fe, Zn, Al, Pb, and Cu all appeared to be of this type. Darlington et al. (1986) placed Cu in type 2 in their study of *Plectrocnemia conspersa* (Curtis) (Trichoptera; Polycentropodidae), and Rainbow and Moore (1986) placed Cu, Fe, Pb, and Zn in type 2 in their study of amphipods (Crustacea: Amphipoda).

The fact that the weight of an organism can play a major part in determining the concentration of metal cations in its tissues has a significant impact upon our attempts to use chemoprinting to discriminate between sources of insects. The implication is that two insects of different weights, coming from different localities or host plants and subject to differing concentrations of metals in their environments, can have identical concentrations of those metals in their tissues. Conversely, insects from the same locality and environment could have different concentrations of metals or ratios of metals by virtue of being of unequal weight. In order to discriminate between insects from different sources, a new variable, dry weight, must be introduced and the relationships between concentrations and dry weight established.

In light of this, it is possible to interpret the study of discrimination of *Noctua pronuba* (L.) (Lepidoptera; Noctuidae) by Bowden et al. (1984). The elements that they measured by XRF were divided into a major group composed of K, P, S, Mg, Ca, and Cl, which could be used for discrimination, and a minor group of Zn, Si, Al, Fe, Cu, Mn, Ni, Cr, and Ti, which could not. The major group metallic cations seem to be of type 1, where weight has little influence, and the minor ones of type 2, where weight is of considerable significance. Their omission of weight as a variable would, therefore, be expected to reduce the reliability of the type 2 elements for discrimination purposes. Indeed we speculate that, since it was possible to use some type 2 metallic cations (e.g., Zn, Fe, and Al) in the study of *Rhopalosiphum padi* (L.) (Homoptera; Aphididae) undertaken by the same authors (Bowden et al., 1985), this may reflect the absence of significant differences in the weight of the individuals in question.

We conclude that an awareness of the effects of the processes of absorption and surface adsorption in the accumulation of metals in individual insects is useful in the development of chemoprinting. Those metals for which absorption is the dominant process of accumulation may be most useful in discrimination, but a wider range of metals is likely to become available for such purposes if the weight of the individual is considered and concentration-weight relationships are used in the analysis.

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SEX PHEROMONE COMPONENTS OF CORN STALK
BORER *Sesamia nonagrioides* (LEF.)¹
Isolation, Identification, and Field Tests

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Abstract—Z-11-Hexadecenyl acetate (Z11-16:OAc), dodecyl acetate (12:OAc), Z-11-hexadecenol (Z11-16:OH), and Z-11-hexadecenal (Z11-16:Ald), were found in pheromone gland extracts of female *Sesamia nonagrioides* (Lef.) [Lepidoptera: Noctuidae]. These four components were also present in airborne volatiles collected from calling virgin females in a 65:18:8:9 ratio. Hexadecyl acetate (16:OAc) was also detected but found to be inactive. The identification was based on multicolumn GC analysis, mass spectrometry, and field activity. Z11-16:OAc is the major sex pheromone component; the addition of the secondary components individually decreased male captures. The blend of the four synthetic components in 69:15:8:8 ratio was highly attractive to males; 200 µg per trap was the most effective concentration in field tests.

Key Words—(Z)-11-Hexadecenyl acetate, dodecyl acetate, (Z)-11-hexadecenal, (Z)-11-hexadecenol, *Sesamia nonagrioides*, Lepidoptera, Noctuidae, sex pheromone.

INTRODUCTION

The corn stalk borer, *Sesamia nonagrioides* (Lef.) [Lepidoptera: Noctuidae], is an important corn pest in many Mediterranean and central African countries (C.I.E., 1979). We reported previously (Mazomenos, 1984) that virgin female abdominal tip extracts attracted males and that the pheromone produced is a multicomponent pheromone. Two pheromone components were identified as

¹Lepidoptera: Noctuidae.

Z-11-hexadecenyl acetate (Z11-16:OAc) and Z-11-hexadecenol (Z11-16:OH) by Sreng et al. (1985). The same authors reported that hexadecyl acetate (16:OAc) was present in the pheromone gland extracts, but its biological activity was not established.

Here we report minor components of the sex pheromone of *S. nonagriodes* that improve male captures in field tests.

METHODS AND MATERIALS

Insects. The insects used in this study were collected as larvae or eggs from infested corn plants. The larvae were separated according to their instar, transferred to artificial diet (Tsitsipis et al., 1983), and allowed to continue their development under laboratory conditions. The temperature was kept at $27 \pm 2^\circ\text{C}$ with a 16:8 hr light-dark regime and relative humidity of $60 \pm 5\%$. The diet was replaced once a week. The eggs were transferred onto a filter paper impregnated with 0.3% propionic acid in Petri dishes for incubation at 27°C . After the four- to five-day incubation period, the emerged larvae were placed on artificial diet. Larval development under the conditions described lasted ca. 30-40 days.

The pupae were kept under the same conditions until moth emergence. Insects were sexed at the pupal stage, five to six days after pupation. The female adults were transferred in an incubator at 25°C with 16:8 hr light-dark regime, while males were placed in a large bioassay cage in a separate room, operated under the same conditions.

Pheromone Collection. The pheromone was collected from the pheromone glands of 2- to 3-day-old virgin females exhibiting calling behavior; the peak of calling occurred in the fifth hour into the dark phase. The glands were removed with forceps and extracted in ether for 20 min. The ether extracts were filtered and concentrated through a 8-cm Vigreux column.

Airborne volatiles from virgin females were collected using a Teckmar GLS-1 closed looping concentrator. Females 2 to 4 days old were placed in the 1-liter sampler during the dark period, and the volatiles emitted from the females were swept by the recirculating headspace gas and absorbed on 1.5 mg of activated charcoal. The trap was then removed and a collection vial was connected to the trap on the end close to the charcoal. Aliquots of $5 \mu\text{l}$ hexane were added twice to the trap. The bottom of the trap-vial assembly was cooled with a Dry Ice cube. This caused the air in the vial to contract, creating a partial vacuum that pulled the solvent through the charcoal. After pulling the solvent down, it was pushed back through the charcoal three times by warming the bottom of the trap with hand contact. The extracts collected were subjected to GC analysis.

Gas Chromatography–Mass Spectrometry. Concentrated crude extracts were injected on a 2-m × 1.8-mm (ID) column packed with 5% Carbowax 20 M on Chromosorb G/HP 80–100 mesh; the column temperature was held at 90°C for 10 min and then was programmed to 180°C at 6°/min. Nitrogen was used as carrier with a flow of 20 cc/min. Consecutive 3-min fractions were collected in liquid-nitrogen-cooled glass capillaries, and each was tested for biological activity in laboratory bioassays. The active fractions recovered from the Carbowax 20 M column were further purified on a 2-m × 1.8-mm (ID) column packed with 3% OV-101 on Chromosorb Q 80–100 mesh operated isothermally at 180°C and collected in 1-min fractions. The active peaks were analyzed on 3% OV-101 (2 m × 1.8 mm ID) on Chromosorb Q 80–100 mesh at 180°C; 5% DEGS (2 m × 1.8 mm ID) on Chromosorb G H/P 80–100 mesh at 160°C; and 15% OV-225 (2 m × 1.8 mm ID) on Chromosorb Q 80–100 mesh at 150°C.

The mass-spectral analyses of the active components were performed using a GC-MS Finnigan MAT 112s spectrometer in the electron-impact mode. The GC glass column was 3.05 m × 0.32 cm (ID), packed with 3.5% OV-101 on Chromosorb Q 100–200 mesh. Helium was the carrier gas at a flow rate of 25 cc/min.

Chemicals. Z11–16:OAc, Z11–16:Ald, and Z11–16:OH were obtained from Sigma Chemical Co. (St. Louis., Missouri); and 12:OAc was synthesized in our laboratory. The components were found to be 96–98% pure when analyzed by GC on OV-101 and OV-225 columns.

Bioassays. Bioassays were conducted in a screen cage described by Mazomenos (1984). Usually 25–40, 1- to 3-day-old males were present in the cage during the bioassay. The bioassays were conducted 5–6 hr into the dark phase under a uniform low level of light (ca. 5 lux). Samples of the crude extract and the fractions obtained during the purification were pipetted onto a piece of Whatman No. 1 filter paper that was suspended about 3 cm below the top of the cage. The response of the males to the sample was observed for 10 min. During each testing period samples were tested individually. The same volume of solvent on filter paper was used as control.

Field Tests. For the field tests conducted during August 13–September 15, 1985, July 3–30, and September 3–October 2, 1986, funnel-type moth traps (Phytophyl, Averof 16, Athens, Greece) were suspended across a corn field in Kopais, central Greece, 1.5 m above the ground, approx. 50 m apart. In 1985, the traps were baited with rubber septa (J12 7 × 11 mm, A.H. Thomas Co., Philadelphia) loaded with 250 µg of each pheromone component and blends containing the Z11–16:OAc and one, two, or three secondary components. In 1986, the traps were baited with different concentrations of the four-component blend. Three replicates for each treatment were used in a complete randomized design. A slow-release formulation of DVP (Vapona) was used as killing agent.

Traps were serviced once a week. Weekly trap catches were subjected to $\log(x + 1)$ transformation prior to statistical analysis. Mean comparisons were made with Duncan's multiple-range test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Gas Chromatographic Analysis. Female pheromone gland extracts were injected on a Carbowax 20 M column and collected in 3-min fractions. The fractions were tested for biological activity in laboratory bioassays. Activity was found in the fractions eluted 9–12 (fraction 4) and 21–24 (fraction 8) min after injection. Further purification of the active fractions on OV-101 column resulted in the isolation of two active components with retention times of 4.3 and 18.7 min for fractions 4 and 8, respectively. The retention times of the two components corresponded to those of synthetic 12:OAc and Z11-16:OAc.

GC-MS analyses of the two components supported the chromatographic data. The mass spectrum for component with a retention time of 4.3 min showed diagnostic peaks of m/z 228 for P and 168 for P-60. This spectrum was identical with that obtained for the synthetic 12:OAc. The mass spectrum for the component with a retention time of 18.7 min established that it was a C₁₆ monounsaturated acetate (m/z 282 for P and 222 for P-60). The double-bond geometry and position are not revealed by the spectrum, but the spectrum was identical to that of synthetic Z11-16:OAc.

These two synthetic components tested separately or in combination in laboratory bioassays produced an activity level less than that elicited from the crude extract. Since Z11-16:OH and 16:OAc have been reported as sex pheromone components (Sreng et al., 1985), we reexamined the pheromone gland extracts and the airborne volatiles collected from virgin females. Three other components were found that had retention times matching those of monounsaturated 16:OH and 16:Ald and saturated 16:OAc. Since the 16:OAc and 16:OH were reported (Sreng et al., 1985) to be of Z-11 configuration, it was assumed that the 16:Ald detected was also of the Z-11 configuration; no other aldehydes were considered. Hexadecyl acetate had no effect on male behavior and was not included in field tests. The ratio of the other four components Z11-16:OAc/12:OAc/Z11-16:Ald/Z11-16:OH was found to be 65:18:8:9, respectively. In laboratory tests, the components Z11-16:OH and Z11-16:Ald, when tested individually, were not attractive to males; however, when the two components were added to the two acetates (Z11-16:OAc and 12:OAc), male attractiveness was increased.

Field Tests. Results of field experiments indicated that Z11-16:OAc attracted quite a high number of males. The blend of the four synthetic com-

ponents in a 69:15:8:8 ratio (similar to that found in the female volatiles) attracted more males than Z11-16:OAc alone (Table 1). The secondary components, 12:OAc, Z11-16:Ald, and Z11-16:OH, when added individually to the Z11-16:OAc in 20, 10, and 10% amounts, respectively, decreased male captures. On the other hand, male capture were increased when two of the three secondary components were added to Z11-16:OAc.

The effect of the pheromone concentration on male captures was studied in 1986, during July with low insect population and in September when the insect population density was high. Concentrations ranging from 50 to 400 $\mu\text{g}/\text{trap}$ were tested, and all attracted males. A trend of higher male attraction was observed in traps baited with 200 μg of the pheromone blend (Table 2).

The results confirmed that Z11-16:OAc is the major sex pheromone component. Many moths of the Noctuidae family are known to use Z11-16:OAc as their major sex pheromone component; *Sesamia inferens* (Nesbitt et al., 1976), *Pseudaletia unipuncta* (Hill and Roelofs, 1980; McDonough et al., 1980; Farine et al., 1981), and *Mamestra brassicae* (Descoins et al., 1978).

Some of these species require the addition of the corresponding alcohol or aldehyde for optimum attraction. The three components were isolated from the species *Mamestra suasa* (Toth et al., 1986) and are essential for male attraction of the cutworm moth, *Crymodes devastator* (Steck et al., 1980). Dodecyl acetate has also been isolated from other lepidopterous species (Inscocoe, 1982), and in most cases this compound acts as a synergist.

In our field tests, traps baited with Z11-16:OAc also attracted a high num-

TABLE 1. MALE *S. nonagrioides* CAPTURED IN TRAPS BAITED WITH DIFFERENT COMBINATIONS OF SYNTHETIC SEX PHEROMONE COMPONENTS, KOPAIS, GREECE, 1985

Z11-16:OAc	Components (μg)			Males caught/ trap/night ^a
	12:OAc	Z11-16:Ald	Z11-16:OH	
225	50	25	25	44.1 ^a
225	50	25	0	27.1b
225	0	25	25	10.6c
250	0	0	0	9.6c
225	50	0	25	2.8d
200	50	0	0	3.6d
225	0	25	0	2.2d
225	0	0	25	4.3d

^aMeans followed by the same letter are not significant different (Duncan's multiple-range test $P = 0.05$).

TABLE 2. MALE *S. nonagrioides* CAPTURED IN TRAPS BAITED WITH VARIOUS CONCENTRATION OF SYNTHETIC SEX PHEROMONE BLEND,^a AT A RATIO OF 69:15:8:8, KOPAIS, GREECE, 1986

Concentration (μg)	Males caught/trap/night ^b	
	Test 1 (July 3–30, 1986)	Test 2 (Sept. 5–Oct. 2, 1986)
50	0.5b	
100	0.8ab	12.5a
150	1.1ab	16.1a
200	1.5a	18.5a
250	0.8ab	13.7a
300	0.9ab	
350	0.8ab	
400	0.4b	

^aZ11-16:OAc/12:OAc/Z11-16:Ald/Z11-16:OH.

^bMeans followed by the same letter in the same column are not significant different (Duncan's multiple-range test, $P = 0.05$).

ber of *P. unipuncta* males (14.7 males/trap/night). Male *P. unipuncta* catches were significantly reduced with the addition of the three secondary components (1 male/trap/night).

The synthetic blend of Z11-16:OAc, 12:OAc, Z11-16:Ald, and Z11-16:OH (69:15:8:8) at a concentration of 200 μg provides a specific attractant for monitoring *S. nonagrioides* population.

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AGGREGATION PHEROMONE OF *Drosophila mauritiana*, *Drosophila yakuba*, AND *Drosophila rajasekari*

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Abstract—(Z)-11-Octadecenyl acetate (Z11-18:Ac) was identified as the aggregation pheromone of *Drosophila mauritiana*, *D. yakuba*, and *D. rajasekari*. The amount of pheromone in the ejaculatory bulb of male flies increased with age, reaching plateau levels of ca. 240, 800, and 1100 ng/fly for *D. mauritiana*, *D. yakuba*, and *D. rajasekari*, respectively. Thirty to 50% of the pheromone in the ejaculatory bulb was transferred to the female during mating, with the majority transferred to the female's reproductive tract. In the subsequent 6-hr period, over half the pheromone in the female's reproductive tract was transferred to the surroundings. In a wind-tunnel olfactometer, Z11-18:Ac was attractive to *D. yakuba* and *D. mauritiana*; however, *D. rajasekari* required food or food odors in combination with Z11-18:Ac to demonstrate aggregation activity. Z11-16:Ac and Z11-20:Ac were not attractive for *D. mauritiana*, *D. yakuba*, and *D. rajasekari*. For all three species, food was synergistic with Z11-18:Ac and both sexes were attracted.

Key Words—*Drosophila mauritiana*, *Drosophila yakuba*, *Drosophila rajasekari*, Diptera, Drosophilidae, (Z)-11-octadecenyl acetate, *cis*-vaccenyl acetate, aggregation pheromone.

INTRODUCTION

Aggregation pheromones have been demonstrated and characterized in species from two subgroups of the *melanogaster* species group (Bartelt et al., 1985; Schaner et al., 1987, 1989a,b). *D. melanogaster* and *D. simulans*, of the *melanogaster* subgroup, were attracted to (Z)-11-octadecenyl acetate (Z11-18:Ac). Z11-18:Ac was produced by mature males, transferred to females during mat-

ing, and emitted by mated females within 6 hr after mating (Bartelt et al., 1985; Schaner et al., 1987). These data suggested that the predominant aggregation pheromone for the *melanogaster* subgroup was Z11-18:Ac.

On the other hand, *D. malerkotliana* and *D. bipectinata*, of the *ananassae* subgroup, were attracted to (Z)-11-eicosenyl acetate (Z11-20:Ac). Z11-20:Ac was produced by mature males, and although they had no Z11-18:Ac, they were as attracted to Z11-18:Ac as to an equal quantity of Z11-20:Ac (Schaner et al., 1989a,b). *D. ananassae*, also of the *ananassae* subgroup, produced mostly Z11-18:Ac but some Z11-20:Ac and was attracted to Z11-18:Ac but not to Z11-20:Ac when tested alone (Schaner et al., 1989b). However, Z11-20:Ac in combination with Z11-18:Ac was significantly more attractive than Z11-18:Ac alone.

Here we demonstrate, identify, and characterize the aggregation pheromone of three additional members of the *melanogaster* group: *D. mauritiana*, *D. yakuba* of the *melanogaster* subgroup, and *D. rajasekari* of the *suzukii* subgroup.

METHODS AND MATERIALS

Flies and Extracts. *D. mauritiana* (strain 14021-0241), *D. yakuba* (strain 14021-0261), and *D. rajasekari* (strain 14023-0361) were obtained from the National *Drosophila* Species Resource Center at Bowling Green, Ohio. The flies were reared on Instant *Drosophila* Medium Formula 4-24 (Carolina Biological Supply Co., Burlington, North Carolina) in 1-liter jars and in 3.5×10 -cm vials under a light-dark cycle of 16:8 hr and ambient laboratory temperatures.

Flies were separated by sex when 0-6 hr old, aged in 3.5×10 -cm food vials to 6-7 days and extracted at that time by soaking the flies in hexane at room temperature for 24 hr. The crude extracts were fractionated on open columns of silicic acid (Bartelt and Jackson, 1984) eluted with hexane; 10% ether in hexane; 50% ether in hexane; and 10% methanol in methylene chloride. Quantitative and preparative gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) were conducted as described previously (Bartelt and Jackson, 1984; Schaner et al., 1989a).

Bioassays. Bioassays were conducted in a wind-tunnel olfactometer (Bartelt and Jackson, 1984) containing ca. 1000 (0- to 2-day-old) flies that had been without food and water for approximately 2 hr. An extract, fraction of an extract, synthetic compound, or control solvent (along with 10 μ l of acetone as a co-tractant for *D. rajasekari*) was applied to filter paper strips inserted around the lip of a glass vial. Each bioassay test consisted of placing two differently treated vials, to be compared, into the olfactometer for 3 min. After 3 min, the vials

were capped and removed, and the captured flies were sexed and counted. Tests were separated by 7–10 min, and as many as 50 tests could be run in a day after stocking the olfactometer once. The bioassay data were transformed to the log ($x + 1$) scale before analysis to stabilize variance, and analysis was done by the method of Yates (1940).

Production, Transfer, and Dispersal of Pheromone. The methods were fully described previously (Schaner et al., 1989a). Briefly, ejaculatory bulbs were removed from adult male flies at various ages, extracted with hexane, and analyzed by GC. The transfer of pheromone during mating was quantitated by removing the reproductive tract of females and the ejaculatory bulb and ducts of males immediately after mating. The organs were crushed with the head of a dissecting pin, extracted with hexane, and analyzed by GC relative to an internal standard.

RESULTS

Identification of Aggregation Pheromone. The hexane extract of 6- to 7-day-old male flies, but not female flies, showed aggregation activity in bioassay (Table 1). Both sexes responded similarly to all preparations tested. Chromatography of the male crude extracts resulted in fractions with some limited activity, but in each case the 10% ether–hexane fraction was equal to or better than the aggregation activity of the crude extract in bioassay (Table 1). Gas chromatography of the 10% ether–hexane fraction revealed that one peak was common to the fraction from all three species. The peak common to each fly was identified as (*Z*)-11-octadecenyl acetate (Z11-18:Ac) by comparing retention times on GC with synthetic Z11-18:Ac (Sigma Chemical Co., St. Louis, Missouri), GC-MS, GC-MS of the dimethyl disulfide reaction product (Nichols et al., 1986), and retention time of synthetic Z9-18:Ac and *E*9-18 = Ac (Sigma Chemical Co.) on GC (Schaner et al., 1989a).

In comparative bioassays, the 10% ether–hexane fraction accounts for all of the aggregation activity of the crude extract (Table 2). When fly-produced Z11-18:Ac (purified from the 10% ether–hexane fraction) was assayed at the same quantity, the Z11-18:Ac accounted for all of the aggregation activity of the 10% ether–hexane fraction. Likewise, synthetic Z11-18:Ac caught the same number of flies as did the crude and 10% ether–hexane fraction.

Pheromone Synergism with Fermenting Food Odors. The combination of synthetic Z11-18:Ac and fermenting food acted synergistically in aggregation activity (Table 3). Fermenting food attracted significantly more flies than Z11-18:Ac alone, but the presentation of Z11-18:Ac along with fermenting food showed 3-, 7-, and 3-fold increases in the bioassay catch for *D. mauritiana*, *D. yakuba*, and *D. rajasekari*, respectively.

TABLE 1. RELATIVE BIOASSAY RESPONSE OF CRUDE HEXANE EXTRACTS OF FEMALE AND MALE ADULT FLIES, AND CHROMATOGRAPHY FRACTIONS OF CRUDE EXTRACT FROM MALE FLIES OF THREE SPECIES OF *Melanogaster* GROUP

Treatment	Relative response in each column ($N = 12$)		
	<i>D. mauritiana</i>	<i>D. yakuba</i>	<i>D. rajasekari</i> ^a
Male crude extract	100*** ^{ab,c}	100***	100***
Hexane fraction	14 (2,2,6) ^{cd}	-3 (1,0.2,18)	-1 (1,1,11)
10% ether fraction	122*** (1,7,6)	101*** (1,16,16)	131*** (2,11,9)
50% ether fraction	25* (3,5,9)	6 (1,6,13)	2 (1,1,9)
10% MeOH-CH ₂ Cl ₂	31* (2,4,9)	6* (1,2,17)	-15 (2,1,12)
Female crude extract	9 (4,5,18)	5* (1,2,18)	-28 (3,2,8)

^a 10 μ l of acetone was used as a coextractant in all treatments for *D. rajasekari*.

^b Relative response calculated from mean bioassay catch of (fraction - control)/(male crude extract - control) \times 100.

^c 100 by definition. ***, and * significant at the 0.001 and 0.05 levels, respectively.

^d Numbers in parentheses are mean bioassay catches for solvent control, treatment, and male crude extract, respectively. Numbers were also rounded to whole numbers for clarity in the table.

TABLE 2. BIOASSAY COMPARISON OF EQUAL QUANTITIES OF Z11-18:Ac IN CRUDE EXTRACT, 10% ETHER-HEXANE FRACTION, MALE-DERIVED Z11-18:Ac, AND SYNTHETIC Z11-18:Ac IN THREE SPECIES OF *melanogaster* GROUP

Treatment ^a	Mean bioassay catch ($N = 24$)		
	<i>D. mauritiana</i>	<i>D. yakuba</i>	<i>D. rajasekari</i>
Male crude extract	5.2 b ^b	30.3 b	13.0 bc
10% ether fraction	6.3 b	25.3 b	14.0 c
Male Z11-18:Ac	5.3 b	23.6 b	11.8 bc
Synthetic Z11-18:Ac	6.8 b	22.7 b	8.9 b
Control	1.6 a	0.9 a	2.1 a

^a All treatments were in hexane solvent. The Z11-18:Ac was at 130 ng, 230 ng, and 300 ng for *D. mauritiana*, *D. yakuba*, and *D. rajasekari*, respectively. Acetone was used as a coextractant for *D. rajasekari*.

^b Means followed by the same letter were not significantly different at the 5% level (LSD).

TABLE 3. SYNERGISTIC ACTIVITY OF AGGREGATION PHEROMONE Z11-18:Ac WITH FERMENTING FOOD ODORS IN WIND-TUNNEL OLFACTOMETER BIOASSAY SYSTEM

Treatment ^a	Mean bioassay catch (N = 24)		
	<i>D. mauritiana</i>	<i>D. yakuba</i>	<i>D. rajasekari</i>
Synthetic Z11-18:Ac	2.5 a ^b	7.8 b	1.3 a
Fermenting food	6.2 b	21.3 c	18.6 b
Z11-18:Ac + food	17.0 c	146.1 d	59.2 c
Control hexane	1.7 a	0.5 a	1.1 a

^aZ11-18:Ac is in hexane solvent at 200 ng, 250 ng, and 300 ng for *D. mauritiana*, *D. yakuba*, and *D. rajasekari*, respectively. The fermenting food is Instant *Drosophila* Medium yeasted for 24 hr.

^bMeans followed by the same letter were not significantly different at the 5% level (LSD).

Chain-Length Specificity in Aggregation Activity. The specificity of the fly's response to certain chain-length Z11-alcohol acetates was tested. Z11-18:Ac was preferred over Z11-16:Ac and Z11-20:Ac (Table 4). Only the Z11-18:Ac was significantly better than the control solvent in attracting *D. mauritiana*, *D. yakuba*, and *D. rajasekari*.

Dose Response of Z11-18:Ac. As the dose of Z11-18:Ac was increased, the mean catch of all three species increased (Figure 1). The response for *D. mauritiana* continued to increase at 250,000 ng of Z11-18:Ac with no indication of a plateau. The response did not increase as rapidly between 2500 and 25,000 ng of Z11-18:Ac for *D. yakuba*, and between 3000 and 30,000 ng of

TABLE 4. SPECIFICITY FOR ACETATE ESTER STRUCTURE IN AGGREGATION BIOASSAY

Treatment ^a	Mean bioassay catch (N = 12)		
	<i>D. mauritiana</i>	<i>D. yakuba</i>	<i>D. rajasekari</i>
Control hexane	1.8 a ^b	1.1 ab	1.6 a
Z11-16:Ac	1.7 a	1.7 b	1.8 a
Z11-18:Ac	7.0 b	7.9 c	12.9 b
Z11-20:Ac	1.9 a	0.7 a	1.1 a

^aThe acetate ester treatments were 200 ng, 700 ng, and 300 ng for *D. mauritiana*, *D. yakuba*, and *D. rajasekari*, respectively. Each treatment for *D. rajasekari* included 10 μ l of acetone as a co-attractant.

^bMeans followed by the same letter were not significantly different at the 5% level (LSD).

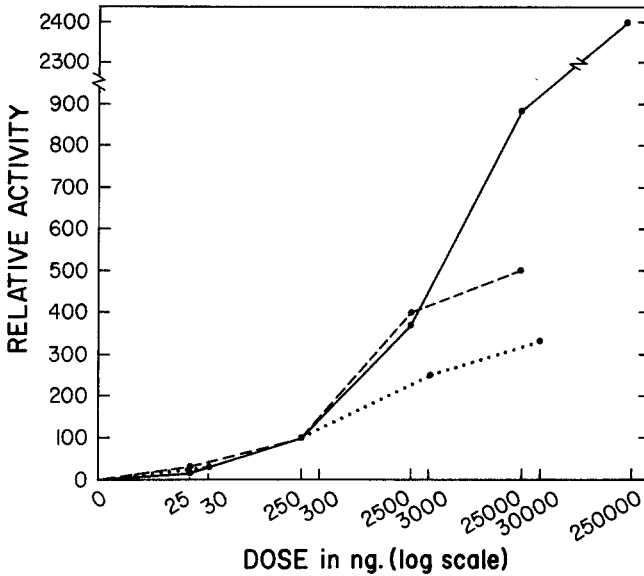


FIG. 1. Dose-dependent aggregation response to the mean Z11-18:Ac content of one mature male fly (relative activity = 100). Solid line for *D. mauritiana*, dashed line for *D. yakuba*, and dotted line for *D. rajasekari*.

Z11-18:Ac for *D. rajasekari*, suggesting that the response might be reaching a plateau (Figure 1).

Location, Transfer, and Release of Z11-18:Ac. In each case, the ejaculatory bulb of male flies contained Z11-18:Ac. With increasing age, there was an increase in Z11-18:Ac in the bulb up to a plateau at five days for *D. yakuba*, and six days for *D. rajasekari* and *D. mauritiana* (Figure 2). The plateau level was ca. 240, 800, and 1100 ng/fly for *D. mauritiana*, *D. yakuba*, and *D. rajasekari*, respectively (Figure 2).

During mating, Z11-18:Ac was transferred from the ejaculatory bulb of the male to the reproductive tract of the female in each species (Table 5). In *D. mauritiana* and *D. yakuba*, ca. 50% of the Z11-18:Ac in the ejaculatory bulb was transferred to the reproductive tract of the female. *D. rajasekari* males transferred ca. 30% of the Z11-18:Ac to the female (Table 5).

In the 6 hr after mating, mated female *D. mauritiana* released nearly 60% of their Z11-18:Ac to the surrounding vial, and mated female *D. rajasekari* and *D. yakuba* released over 50% of their Z11-18:Ac to the surroundings (Table 5). The amount of Z11-18:Ac on the fly minus the reproductive tract remained nearly constant. Mated male flies of all three species and virgin male *D. mauritiana* released some Z11-18:Ac to the surroundings.

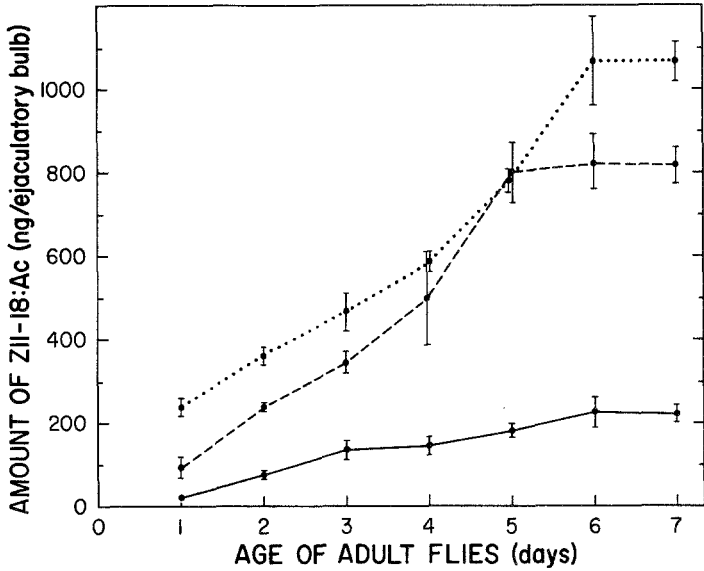


FIG. 2. Z11-18:Ac content in the ejaculatory bulb of male flies as they age. Solid line for *D. mauritiana*, dashed line for *D. yakuba*, and dotted line for *D. rajasekari*.

TABLE 5. TRANSFER OF Z11-18:Ac FROM EJACULATORY BULB OF MALE TO FEMALE DURING MATING AND RELEASE TO SURROUNDINGS 6 HR AFTER MATING

Flies	Z11-18:Ac (ng/fly) ^a		
	<i>D. mauritiana</i>	<i>D. yakuba</i>	<i>D. rajasekari</i>
During mating			
Virgin male ej. bulb ^b	234	591	676
Mated male ej. bulb	110	334	478
Mated female repro. tract ^b	94	208	139
Remainder of fly ^d	20	64	50
Virgin female repro. tract	0	0	0
(6 hr after mating)			
Mated female repro. tract	8	80	54
Remainder of fly	11	62	10
Vial ^e	51	126	63
Mated male ej. bulb	60	261	310
Vial	43	89	64

^a $N \geq 2$ groups of five or more flies.

^bej. bulb = ejaculatory bulb.

^crepro. tract = reproductive tract less the ovaries.

^dExtract of the remainder of the fly with reproductive tract removed.

^eExtract of the glass vial in which the fly spent the 6 hr.

DISCUSSION

The production of Z11-18:Ac and the specific response to Z11-18:Ac by *D. mauritiana* and *D. yakuba* are consistent with the concept that the aggregation pheromone of the *melanogaster* subgroup is Z11-18:Ac. The remainder of the *melanogaster* subgroup is yet to be investigated, but a GC peak corresponding to Z11-18:Ac has been observed from extracts of male *D. sechellia* (Luyten, 1982), suggesting that all species of the *melanogaster* complex produce Z11-18:Ac. *D. rajasekari* (of the *suzukii* subgroup) is like the members of the *melanogaster* subgroup in that they produce and respond specifically to Z11-18:Ac.

In the species of the *melanogaster* group, there is considerable variation in the amount of Z11-18:Ac in the ejaculatory bulb of sexually mature male flies: *D. melanogaster* has ca. 1400 ng/fly (Bartelt et al., 1985), *D. rajasekari* has ca. 1000 ng/fly, *D. simulans* (Schaner et al., 1987) and *D. yakuba* have ca. 800 ng/fly, and *D. mauritiana* has ca. 240 ng/fly of Z11-18:Ac.

A comparison of the amount of Z11-18:Ac transferred to the female during a complete mating shows that *D. melanogaster* (Bartelt et al., 1985) and *D. simulans* (Schaner et al., 1987) each transferred ca. 420 ng/fly of Z11-18:Ac to the female whereas *D. yakuba* and *D. rajasekari* transferred ca. 230 ng/fly, and *D. mauritiana* transferred ca. 120 ng/fly. All of the flies transferred the bulk of the Z11-18:Ac into the reproductive tract of the female, and she subsequently transferred the Z11-18:Ac to the surroundings. The mechanism of transfer of the aggregation pheromone to the surroundings by mated females is unknown; but the majority of the transfer occurs in the first 6 hr after mating (Bartelt et al., 1985; Schaner et al., 1987, 1989a). In contrast, both mated and virgin *D. ananassae* males released more Z11-18:Ac to the surroundings (ca. 38 ng/fly) than mated females did (5 ng/fly) in a 12-hr period, suggesting that males are the primary releasers of aggregation pheromone for *D. ananassae* (Schaner et al., 1989b).

There does not appear to be a plateau of maximum dose of Z11-18:Ac for aggregation response in the members of the *melanogaster* subgroup so far tested. The dose-response curve did not plateau up to 10,000 ng for *D. simulans* (Schaner et al., 1987), up to 25,000 ng for *D. yakuba*, and up to 250,000 ng for *D. mauritiana*.

In summary, aggregation pheromone activity has been demonstrated in *D. mauritiana* and *D. yakuba* of the *melanogaster* subgroup and in *D. rajasekari* of the *suzukii* subgroup of the *melanogaster* species group. (Z)-11-Octadecenyl acetate was attractive alone in *D. mauritiana* and *D. yakuba* but required food odors to be attractive in *D. rajasekari*. The pheromone can be isolated from the ejaculatory bulb of sexually mature male flies but not from virgin female flies. During mating, there is a transfer of the pheromone to the female's reproductive

tract. Within 6 hr after mating, the females release the majority of the pheromone to the surroundings where it could synergize the attraction of food odors for flies of both sexes.

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COMPARISON OF SEX PHEROMONE COMPOSITION
AND PHEROMONE-MEDIATED MALE BEHAVIOR
OF LABORATORY-REARED AND WILD
Heliothis zea (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Analysis of ovipositor extracts of lab-reared (> 120 generations) and wild *Heliothis zea* indicated small but significant differences in the percent composition of the four aldehyde components of the sex pheromone. (Z)-11-hexadecen-1-ol (Z11-16:OH) was present in both populations and amounted to only 2.8% of the total pheromone. There was no significant difference in four of the six behavioral categories between lab-reared and wild males exposed to the four-component sex pheromone in the flight tunnel. Ninety and 84% males flew to the pheromone source, respectively. However, with the addition of Z11-16:OH to the pheromone blend, none of the wild males flew upwind to the stimulus source, whereas 36% of the lab-reared males completed the flight. It is suggested that prolonged inbreeding in closed quarters rendered these males less discriminating to qualitative differences in a pheromone blend.

Key Words—Corn earworm, *Heliothis zea*, Lepidoptera, Noctuidae, pheromone, (Z)-11-hexadecen-1-ol, laboratory rearing.

INTRODUCTION

Ovipositor extracts of the corn earworm, *Heliothis zea* (Boddie), Klun et al. (1979, 1980a) were reported to contain four 16-carbon aldehydes as components of its sex pheromone. Recently, Teal et al. (1984) reported the presence

of (*Z*)-11-hexadecen-1-ol (*Z*11-16:OH) in the pheromone gland extracts of *H. zea* females. However, Pope et al. (1984) did not detect *Z*11-16:OH in volatile collections made from *H. zea*. To the best of our knowledge, all previously reported pheromone identifications of *H. zea* were carried out on insects that had been in laboratory culture on meridic diets for many generations. The present study was conducted to determine if prolonged laboratory rearing caused any changes in the qualitative composition of the female sex pheromone and male behavioral responses to pheromonal stimuli.

METHODS AND MATERIALS

All experimental insects were obtained from the Southern Field Crop Insect Management Laboratory, Stoneville, Mississippi. Laboratory-reared insects had been in culture on a wheat-germ diet for over 120 generations under a 15:9 hr light-dark photoperiod (the culture was originally started at the Southern Grain Insects Research Lab in Tifton, Georgia, and subsequently subcultured at Stoneville, Mississippi). These insects were shipped to Beltsville as first-instar larvae on the wheat-germ diet. Wild insects were collected as larvae from cotton, in the vicinity of Stoneville, fed cotton squares and bolls, and shipped as mature larvae. At Beltsville, the insects were reared in environmental chambers maintained under a reverse 16:8 hr light-dark photoperiod and temperatures of 26°C and 22°C during the light and dark phases, respectively. Pupae were sexed and placed in separate chambers. Adults were provided a 10% sucrose solution. Wild *H. zea* females produced a negligible amount of pheromone unless provided with a host plant (Raina, 1988). On the other hand, lab-reared females did not require the presence of a host plant for pheromone production. In order to avoid any discrepancy, females of both populations were provided with cotton squares in the cages.

Pheromone extractions were made from ovipositors of single females in their third scotophase (54 hr posteclosion) as described by Raina et al. (1986a,b). The extracts were analyzed for the five known components using the internal standard method of quantitative analysis. Instrumentation and chromatographic conditions were the same as described by Raina et al. (1986a).

Behavioral response of lab-reared and wild males to four-component [(*Z*)-11-hexadecenal (*Z*11-16:Ald), (*Z*)-7-hexadecenal (*Z*7-16:Ald), (*Z*)-9-hexadecenal (*Z*9-16:Ald), and hexadecanal (16:Ald)] and five-component (the above four plus *Z*11-16:OH) synthetic blends was tested in a flight tunnel previously described by Raina et al. (1986c). Synthetic pheromones containing 230 ng of *Z*11-16:Ald (the percentage of *Z*11-16:Ald, *Z*7-16:Ald, *Z*9-16:Ald, 16:Ald, and *Z*11-16:OH was 91.4, 0.6, 1.5, 3.8, and 2.7, respectively) were applied to 4 × 0.5-cm filter paper strips. A freshly treated strip was used for each replicate. In a preliminary flight-tunnel test, conducted at a light intensity of 5

lux (from red incandescent bulbs), the wild males showed no response to the pheromone, whereas the lab-reared males responded with normal behavior. Subsequently, it was determined that wild males would respond only if the light intensity was < 2 lux. All the tests were therefore conducted at 0.5 lux red light. Male responses for the following six behavioral categories were monitored: (1) activation (rapid body movement and wing fanning); (2) time to activation (elapsed time from exposure to pheromone to start of the wing fanning); (3) upwind flight in plume (divided into two subcategories: (a) in which the males followed the pheromone plume to its source, and (b) in which the males flew only part of the distance and then flew out of the plume); (4) flight time (time from commencement of the flight in plume to arrival at the source); (5) copulatory attempt (represented by the males that flew upwind to the source, landed on it, and attempted copulation as visualized by extension of the claspers and bending of the abdomen); and (6) time spent by the males on the source. Forty-five males from each population were tested for each pheromone treatment.

Percentages of each component of the sex pheromone were statistically analyzed by *t* test for differences between lab-reared and wild females. The time variables were analyzed after log transformation. Time to activation was analyzed as a 2×2 factorial, other time variables were analyzed by one-way analysis of variance, since wild males did not fly to the five-component blend. Frequency variables were analyzed by 2×4 or 2×3 contingency table analysis depending on whether there were three or four variables.

RESULTS

Pheromone Composition. Gas chromatographic analyses of heptane extracts of ovipositors from both lab-reared and wild females showed five peaks whose retention times on polar and apolar columns were coincident with authentic Z11-16:Ald, Z7-16:Ald, Z9-16:Ald, 16:Ald, and Z11-16:OH. The average amount of Z11-16:Ald produced by wild and lab-reared females was 102.5 and 117.8 ng/female. The extracts from wild females contained a significantly higher percentage of Z11-16:Ald than did the extracts from lab-reared females. However, the extracts from lab-reared females contained a significantly higher percentage of the remaining three aldehydes when compared to those from the wild (Table 1). The quantity of Z11-16:OH was 2.8% in extracts from both types of females.

Male Behavior. All test males were activated when presented with either four- or five-component pheromone blends. Lab-reared males were activated in significantly less time than wild males in response to both the four- and five-component blends (Table 2). There was no significant difference in the percentage of the lab-reared and wild males that flew upwind to the four-compo-

TABLE 1. COMPOSITION OF SEX PHEROMONE EXTRACTED FROM *Heliothis zea* FEMALES IN LABORATORY CULTURE AND REARED FROM FIELD-COLLECTED LARVAE

Sex pheromone component	Composition (%) ^a	
	Lab-reared (N = 15)	Wild (N = 20)
(Z)-11-Hexadecenal	90.2 ± 0.52 (87.2–92.6)	92.5 ± 0.45 (89.2–97.0)* ^b
(Z)-7-Hexadecenal	0.7 ± 0.05 (0.2–1.2)	0.4 ± 0.05 (0.2–0.8)*
(Z)-9-Hexadecenal	2.0 ± 0.20 (0.5–3.6)	1.2 ± 0.17 (0.3–2.5)*
Hexadecanal	4.4 ± 0.38 (2.6–8.5)	3.2 ± 0.33 (1.4–6.0)*
(Z)-11-Hexadecen-1-ol	2.8 ± 0.35 (1.2–5.2)	2.8 ± 0.30 (0.9–5.6) ns

^aMean ± standard error and (range).

^b* Means within a row are significantly different at 5% level of probability by *t* test.

ment source; 89% and 84% of the respective males completed the flight. The addition of the alcohol resulted in a significant decrease in the percentage of lab-reared males that flew to the source, and none of the wild males completed the flight. A significantly higher percentage of lab-reared males flew part of the distance in the five-component pheromone plume as compared to the wild males (Table 2). Lab-reared males took significantly longer to complete the flight to

TABLE 2. BEHAVIORAL RESPONSE OF LAB-REARED AND WILD *Heliothis zea* MALES TO 4- and 5-COMPONENT SEX PHEROMONE IN FLIGHT TUNNEL

Behavioral category	Four component ^a		Five component ^b	
	Lab-reared	Wild	Lab-reared	Wild
Activation (%)	100.0	100.0	100.0	100.0
Time to activation ^c	2.7 c	8.8 b	7.7 b	14.8 a
Upwind flight in plume (%)				
To the source	88.9 a	84.4 a	35.6 b	0.0 c
Part of the way	8.9 b	8.9 b	46.7 a	4.4 b
Flight time ^d	17.5 b	17.0 b	23.1 a	—
Landing and attempted copulation (%)	82.5 a	78.9 a	50.0 b	—
Time on source	20.3 a	12.0 b	17.4 ab	—

^aZ11-16: Ald, 230 ng; Z7-16: Ald, 1.4 ng; Z9-16: Ald, 3.9 ng; and 16: Ald, 9.5 ng.

^bZ11-16: OH, 6.9 ng; in addition to the four components in *a*.

^cAll time measurements are in seconds.

^dData in this and subsequent categories are based on those males that flew upwind in plume to the source. Values in a row followed by the same letter are not significantly different from each other (see text for description of statistical procedures.)

the five-component blend as compared to the four-component blend. There was no statistically significant difference in the number of lab-reared and wild males that, having flown to the four-component source, landed on it and attempted copulation. Of the lab-reared males that flew to the five-component source, only 50% landed and attempted copulation. Time spent by the lab-reared males on the four-component source did not differ significantly from that spent on the five-component source. Wild males spent significantly less time on the four-component source as compared to the lab-reared males.

DISCUSSION

Prolonged lab rearing did not appear to change the qualitative composition of *H. zea* female sex pheromone. The percentage of each of the four aldehydes was similar to that reported by Klun et al. (1980a). The presence of Z11-16:OH in ovipositor extracts of *H. zea* females, representing 27.9% of the total pheromone was reported by Teal et al. (1984). However, we observed a much smaller proportion of this component. The difference may be due to the highly polar solvent (ethyl ether) that was used by Teal and coworkers for their extractions compared to the less polar heptane that we used. Z11-16:OH has been reported in the ovipositor extracts of all *Heliothis* species for which the sex pheromones have been identified (Klun et al., 1980b, 1982; Nesbitt et al., 1979; Raina et al., 1986a). However, only in the case of *H. phloxiphaga*, was Z11-16:OH shown to be a component essential for the elicitation of male response (Raina et al., 1986a). In *H. zea*, Teal et al. (1984) speculated that Z11-16:OH may be an immediate precursor of the corresponding aldehyde. This may also explain why Z11-16:OH was not detected in the volatiles collected from calling *H. zea* females (Pope et al., 1984).

In contrast to minor changes in pheromone blend quality, the behavioral responses of lab-reared males were markedly different from those of the wild males. The requirement of very low light intensity (0.5 lux red light) for the wild males to respond to the sex pheromone reflects the conditions in their natural habitat. Even on full-moon nights the light intensity is no greater than 0.3 lux (Shorey and Gaston, 1964). In *Trichoplusia ni*, for example, the males required 10,000-fold more pheromone at 30 lux to elicit behavioral response equivalent to that obtained at 0.3 lux (Shorey and Gaston, 1964). Lab-reared *H. zea* males appear to have lost the requirement for very low light intensity as a prerequisite for them to be able to respond to the sex pheromone.

The most important single parameter in the behavioral repertoire of males in response to the sex pheromone is the upwind flight to the source. It was very evident that the addition of Z11-16:OH caused a complete obliteration of this response in the wild males. However, about 82% of lab-reared males initiated upwind flight in pheromone plume and about 36% made it to the source. Of

those that flew to the source, only 50% landed and attempted copulation. In a field study, Teal et al. (1984) reported that blends containing 1% or more of Z11-16:OH reduced trap capture of *H. zea* to that of unbaited traps. All these results suggest that even though Z11-16:OH is present in *H. zea* female ovipositor extracts, it is not a component of the sex pheromone that is released by the females. In fact, Z11-16:OH in *H. phloxiphaga* sex pheromone is very important in maintaining sex pheromone specificity between this species and *H. zea* (Raina et al., 1986a).

Prolonged rearing appeared to have adversely affected the ability of *H. zea* males to discriminate the presence of Z11-16:OH in the pheromone blend. It is, therefore, very important in evaluating the role of various components in the sex pheromone of a species, either to conduct a parallel field test or to use insects that have not been in laboratory culture for extended periods.

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CHEMICAL COMPOSITION OF DEGRADING
MANGROVE LEAF LITTER AND CHANGES
PRODUCED AFTER CONSUMPTION BY
MANGROVE CRAB *Neosarmatium smithi*
(Crustacea: Decapoda: Sesarmidae)

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Abstract—The leaves of the mangrove *Ceriops tagal* contained 3.2–4.1% (all percentages relate to dry weight) of D-1-*O*-methyl-*muco*-inositol previously unreported in mangroves. They consisted of 37% aqueous acetone-water-soluble material, 18% water-insoluble polysaccharides, and ca. 50% polyphenols, which include soluble and insoluble tannins and lignin. The polysaccharide component sugars were glucose, arabinose, uronic acids, mannose, xylose, galactose, and rhamnose in the proportions 28:26:22:10:7:5:2, respectively. The leaves were pectate rich, and the low level of glucan was presumed to consist mainly of cellulose. After four weeks of biodegradation, ca. 60% of the acetone-water-soluble material was lost from the leaves. Degradation processes greatly altered the polysaccharide components in the leaves. Pectates were rapidly degraded, while other polysaccharides, although reduced proportionately, resisted degradation at about the same level, and all component sugars were found in the 8-week-old leaves. “Apparent lignin” contents increased from 15 to >30% during biodegradation up to eight weeks. The yields of the major fractions in corresponding fecal material from *Neosarmatium smithi* showed a similar trend to the diets. An enrichment of the insoluble residue was noticeable due to the digestion of dialyzable material. The fecal carbohydrate content was greatly reduced (7–11%) and the “apparent lignin” increased (27–39%) due to its resistance to degradation. All dietary polysaccharide component sugars were found in the

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fecal residues, including some uronic acids. The leaves also contained a readily water-soluble fraction (15%) which consisted of pectates strongly complexed with proanthocyanidins.

Key Words—Mangrove, detritus, tannins, carbohydrates, polysaccharides, D-1-O-methyl-muco-inositol, *Neosarmatium smithi*.

INTRODUCTION

The degradation of mangrove leaf litter is a dominant factor in the ecology of mangrove swamps, and the detritus is an essential nutrient source for the ecosystem food chain (Odum and Heald, 1975). For example, litter may be decomposed by microorganisms (Bunt et al., 1979; Cundell et al., 1979; Benner and Hodson, 1985) and crustacea (Malley, 1978; Leh and Sasekumar, 1985; Giddins et al., 1986; Poovachiranon et al., 1986; Robertson, 1986).

Detailed chemical studies have been made on leaf composition with respect to its elements (Spain and Holt, 1980; Kotmire and Bhosale, 1980), organics—lipids, sterols, hydrocarbons (Hogg and Gillan, 1984; Ghosh et al., 1985), and low-molecular-weight carbohydrates (Popp, 1984). Other studies (Jamale and Joshi, 1976; Bhosle et al., 1976; Sumitra-Vijayaraghavan et al., 1980) have used proximate analyses only to obtain information about leaf composition and decomposition. However, there have been few specific studies (Benner et al., 1984) on the detailed chemistry of mangrove leaf cell walls, in particular the polysaccharides and their primary degradation processes. Understanding the factors that control the degradation of the walls is of great ecological significance.

Plant cell walls inherently resist digestion in animals, due largely to their water-insoluble constituents. For example, only half the wall polysaccharides are digested during passage through a ruminant (Dekker et al., 1972). This study looks at specific chemical changes occurring in leaf litter degrading at the mud surface (Giddins et al., 1986) and also provides information on the detailed composition of undigested leaf material after passage through the mangrove crab *N. smithi* (Crustacea: Decapoda: Sesarmidae).

Interest also focused on the low-molecular-weight carbohydrates (or free sugars), since they arguably play a significant role in osmotic adjustment in halophytes such as mangroves (Flowers et al., 1977; Popp, 1984).

METHODS AND MATERIALS

General. The conditions for drying samples were: 1 mm Hg, P₂O₅, <40°C, 16 hr. All dialysis tubing, molecular weight (mot wt) cutoff 12,000–14,000, was thoroughly preextracted with distilled water throughout these

experiments. Total phenolics were measured using Folin-Ciocalteu reagent (Hartree, 1972). Condensed tannins were estimated by the vanillin HCl (Broadhurst and Jones, 1978) method with catechin as a reference. Where necessary, carbohydrates were estimated colorimetrically using methods as applied in Neilson et al. (1986a). In the phenol-sulfuric acid method, values were corrected for any absorbance at 485 nm produced by a blank where the phenol reagent was replaced by water (see later).

Sodium chloride in leaves was estimated from sodium determinations made by atomic absorption spectrometry (AAS) with a Varian Techtron atomic absorption spectrophotometer AA6. Milled (<40 mesh) samples (ca. 100 mg) were extracted with double-distilled water (300 ml), first with stirring at 70°C for 30 min (Birch, 1975) and second with sonication for 30 min. Samples were vacuum filtered, residues washed, and the filtrates and washings made up to 500 ml for analysis by AAS.

Samples and Feeding Experiments. Mature green and senescent leaves from *C. tagal*, the collection of senescent leaves subjected to biodegradation for zero to eight weeks (S2, etc. . . . , two-week biodegradation, etc. . . .) and feeding experiments with *N. smithi* are fully described in Giddins et al. (1986).

Fractionation Procedures. Initially leaf fractions were isolated as outlined by Blake and Richards (1970). However, a more direct approach using acetone and (ethylenedinitrilo)tetraacetic acid (EDTA) or water extractions was favored for the bulk of this work (Neilson et al., 1986a). The procedure was modified (Giddins et al., 1986) in order to remove microbial and mud contamination from S2 to S8 leaves. Acetone-insoluble material was dried and weighed and combined acetone extracts were filtered, concentrated to dryness, and weighed. Corresponding fecal samples F2 to F8 (Giddins et al., 1986) were treated similarly but without the homogenization and sonication steps. The proanthocyanidin/polysaccharide complex initially described in Neilson et al. (1986a) was present in the EDTA-soluble fraction.

Dried acetone extracts (0.1 g) and also acetone-insoluble fractions (0.1 g) were extracted with absolute methanol (5 ml) (Ribereau-Gayon, 1972a) for 1 hr at 30°C. Extracts were then centrifuged and total phenolics and condensed tannins measured.

Analysis of Free Sugars. The dried aqueous ethanol soluble fraction (6.1 g) (see Table 2 below), was extracted with water (3×10 ml). Centrifugation (20,000g for 30 min) was needed to remove insoluble material from the first extract, while the two subsequent extracts were centrifuged 2000g for 10 min. Pellets were recovered and represented 27% of the total fraction. The clear reddish brown supernatants were combined and deionized using tandem columns of Amberlite resins IR 120 (H) and then IR 45 (OH) (100 ml of each). The neutral eluent was concentrated to dryness and weighed (37% of the fraction). The resins removed 36% of the fraction, about half of which was sodium chloride.

High-performance liquid chromatography (HPLC) was used to quantify the free sugars in 5% w/v solutions of the deionized water-soluble material. The conditions were: eluent, water-acetonitrile, 30:70, v/v; flow, 1.5 ml/min; temperature, 25°C; column, Radial-PAK L.C. cartridge μ Bondapak NH₂ (8 mm \times 10 cm) (Waters Associates, USA). Fructose and glucose were poorly resolved under these conditions. For preparative HPLC, water-acetonitrile, 25:75, v/v; 3 ml/min and 100 μ l injections of sample solution were used.

Thin-layer chromatography (TLC) was on cellulose sheets (Merck) with ethyl acetate-pyridine-water-*n*-butanol-acetic acid, 5:4:4:10:2, v/v as the solvent (Hoton-Dorge, 1976). Two detection systems were used: (1) silver nitrate-sodium hydroxide (Trevelyan et al., 1950; Hough and Jones, 1962), and (2) *p*-aminohippuric acid (Hoton-Dorge, 1976). Sheets were eluted three times with intermediate drying.

For the gas-liquid chromatography (GLC) of TMS derivatives of carbohydrates, the conditions were: column, 3% SE-30 on Chrom W-HP 80-100; 100°C + 10°C/min to 250°C. For the derivatization with *N*-trimethylsilylimidazole (Pierce, USA) reaction temperature (60-80°C) and time (5-150 min) varied according to solubility of standards and samples. Inositol was used as the internal standard.

Nuclear magnetic resonance (NMR) spectra of solutions in deuterium oxide (5% w/v) were recorded (δ) with a JEOL JNM-MH 100 MHz spectrometer at 29°C with acetone and sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal references.

The optical rotation was read with a Perkin-Elmer model 141 polarimeter.

Polysaccharide and "Apparent Lignin" Analysis of Fractions. Fractions were subjected to total acid hydrolysis based on modified Saeman conditions (Saeman et al., 1954), prior to assaying. Samples (15-25 mg) were weighed into a tared centrifuge tube (10 ml), triturated with 72% w/w sulfuric acid (100 μ l) at 30°C, and kept in a desiccator with occasional stirring for 1 hr. Water was added (2.8 ml), and the tube and contents were tared prior to secondary hydrolysis under reflux for 3 hr at 100°C. The mixture was cooled, weight was adjusted with water (if necessary), mixed, centrifuged (1000g, 10 min), and aliquots were taken for phenol-sulfuric assay and uronic acid measurement. The remaining known volume was then transferred to a second centrifuge tube and neutralized (pH paper) with barium carbonate at 60°C. After cooling, methyl α -D-glucopyranoside (2%, 0.25 ml) was added as internal standard, the tube contents were thoroughly mixed, and the precipitated salts were removed by centrifugation.

The glyucose composition in the neutralized acid hydrolysates was determined by GLC of the derived alditol acetates. A Hewlett-Packard 5890A gas chromatograph was used with flame-ionization detection and fitted with a vitreous-silica capillary column (BP20, 0.22 μ m, 12 m \times 0.22 mm, S.G.E., Mel-

bourne, Australia). Conditions were: oven 235°C, injector 250°C, detector 300°C, helium carrier gas 0.43 ml/min, split ratio 1:85. A newly developed column (BP225, 0.1 μ m, 25 m \times 0.22 mm, S.G.E.) was also used for separation of alditol acetates isothermally at 220°C with hydrogen carrier gas at 40 cm/sec and with either inositol or allose (3 mg) added immediately after secondary hydrolysis as the internal standard.

The neutralized hydrolysate (0.5 ml) in a 40-ml test tube was reduced with sodium borohydride (2% w/v, 0.5 ml) for 90 min at 30°C. The excess borohydride was destroyed by adding glacial acetic acid dropwise until evolution of hydrogen was complete. The solution was cooled to 0°C and *N*-methylimidazole (2 ml) (McGinnis, 1982) and acetic anhydride (6 ml) were added, the latter with caution. After 5 min, the reaction mixture was cooled, chloroform (5 ml) added, and the solution washed with water (2×5 ml). The organic phase was dried with anhydrous sodium sulfate, evaporated to dryness in a stream of nitrogen, and redissolved in acetone (0.5 ml) for GC.

Peaks were identified by cochromatography with added authentic sugars and quantified as anhydro sugars using a Hewlett-Packard 3392A integrator-recorder, using response factors relative to the internal standard derived from standard sugars subjected to the total analytical procedure including hydrolysis.

The insoluble residues produced on acid hydrolysis were washed free of acid with water (3×10 ml), vacuum dried, and weighed. These crude lignin samples were ashed (600°C, 4 hr), and the weight was adjusted for the ash yield and reported as "apparent lignin."

The protocol used here to analyze plant cell walls allows neutral sugars, uronic acids, and apparent lignin to be determined simultaneously and permits a mass balance to be made, which may be used to check the accuracy of the overall method. The total acid hydrolysis conditions of primary hydrolysis at 30°C for 1 hr, dilution to 0.41 M sulfuric acid, and then 3 hr at 100°C are critical for the quantitative generation of carbohydrate monomers from wall polysaccharides. Using correction factors (relative response factors) derived from subjecting authentic sugars to the total analytical procedure, including hydrolysis, the reproducibility of the procedure is demonstrated in Table 1. Three mixtures each containing seven sugars produced factors that had standard deviations of $\leq \pm 0.079$, $N = 3$. Furthermore, Avicel cellulose was $>90\%$ recovered as glucose when analyzed by the method.

RESULTS AND DISCUSSION

Green (66% moisture) and senescent (68% moisture) leaves were first fractionated using a classical approach for isolation of plant polysaccharides (Blake and Richards, 1970). Plant material was isolated and separated sequentially into

TABLE 1. PRECISION OF RELATIVE RESPONSE FACTORS IN ALDITOL ACETATE PROCEDURE RELATIVE TO METHYL α -D-GLUCOPYRANOSIDE (INTERNAL STANDARD) AS UNITY

Sugar	Mixture			Average \pm standard deviation
	1	2	3	
Rhamnose	2.210	2.113	2.270	2.198 \pm 0.079
Fucose	1.714	1.627	1.747	1.696 \pm 0.062
Arabinose	0.983	0.884	0.994	0.954 \pm 0.061
Xylose	1.068	0.987	1.106	1.054 \pm 0.061
Me- α -glucose	1.000	1.000	1.000	1.000 \pm 0.000
Mannose	1.182	1.180	1.095	1.152 \pm 0.041
Galactose	1.113	1.165	1.047	1.108 \pm 0.059
Glucose	1.250	1.296	1.184	1.243 \pm 0.056

operationally defined fractions, namely, hot water (HW), cold water (CW), ammonium oxalate (pectic substances), chlorite (removes lignin, which produces a cellulose and hemicelluloses fraction) and sodium hydroxide (hemicelluloses) (Table 2). Apparent lignin was also measured using 72% sulfuric acid (Adams, 1965). Similar fraction yields were obtained for the two types of leaves except for CW extracts (9.1 vs. 16.9%) and lignin (17.9 vs. 12.1%). Since both CW extracts contained similar proportions of carbohydrate (6.2 vs. 5.4%

TABLE 2. DISTRIBUTION OF COMPONENTS IN MATURE GREEN AND SENESCENT LEAVES OF *Ceriops tagal* OBTAINED BY TRADITIONAL FRACTIONATION PROTOCOL (BLAKE AND RICHARDS, 1970)

Fraction	Leaf dry wt (%)	
	Green	Senescent
80% ethanol soluble ^a	38.8	36.7
Chloroform soluble	0.5	0.2
Cold water soluble	9.1	16.9
Hot water soluble	4.6	5.3
Pectic substances	3.8	6.3
Lignin	17.9	12.1
Hemicelluloses A	0.2	0.6
Hemicelluloses B	4.6	4.3
α -Cellulose	10.6	8.7

^aIncludes low-molecular-weight carbohydrates (free sugars), salt(s), and water-insoluble material.

of leaf dry weight), this major difference could only be attributed to a change in the noncarbohydrate, predominantly proanthocyanidin component (see later). The major fraction was obtained as the 80% ethanol extract (ca. 37%), and this material was analyzed for free sugars.

Bonded-phase HPLC analysis of the water-soluble extract from 80% ethanol-soluble material showed two well-resolved peaks, I and II (area ratio 4:1), which coeluted with fructose and sucrose standards, respectively. TLC using detection systems 1 and 2, confirmed the presence of sucrose but not fructose or glucose. Both systems detect fructose, but, although system 1 showed apparent fructose in the sample, system 2 did not. GLC analysis of the TMS derivatives of the water-soluble components showed two components: sucrose and a compound almost coeluting with authentic fructose, retention time relative to inositol (R_{it}), 0.79 vs. 0.78.

Following isolation by preparative HPLC, [^1H]NMR spectroscopy confirmed that peak I was not fructose. The presence of a singlet resonance at δ 3.40 indicated a methoxyl group, and the remaining partially resolved resonances at δ 4.03–3.54 suggested that peak I may be a monomethyl cyclitol (Angyal and Odier, 1983) and in particular 1-*O*-methyl-*muco*-inositol (Angyal and Kondo, 1980). Integration of the spectrum supported this identification. A [^1H]NMR spectral comparison showed that peak I was not pinitol, which has a methoxyl group shift of δ 3.59. Furthermore, there was little similarity in the respective ring proton patterns. [^{13}C]NMR spectroscopy of peak I with complete decoupling showed that the molecule had a plane of symmetry. Chemical shifts relative to acetone at δ 30.6 were observed at: 82.1 (C-OCH_3), 72.6 ($2 \times \text{C}$), 70.4, 70.2, 68.5, and 58.1 (OCH_3). Peak I was observed to be optically active $[\alpha]_D^{20} = -25.2^\circ$ (c 0.28, water), and these combined data suggested that the unknown compound was (-)-1-*O*-methyl-*muco*-inositol (MMI) (Angyal et al., 1967). Comparative [^{13}C]- and [^1H]NMR spectroscopy with authentic MMI gave unequivocal identification.

Quantitative HPLC analysis with relative responses determined from authentic compounds gave only MMI and sucrose as 3.2% and 0.8% of the total dry weight of fresh green *C. tagal* leaves. Senescent leaves were shown to contain MMI (4.1%) and sucrose (0.8%); a minor peak with retention time relative to MMI (0.80) was also observed but not identified.

MMI has been found in most gymnosperms and some angiosperms (Dittrich et al., 1971). Its occurrence has not been reported in mangrove species, although a significant amount of pinitol has been reported in various mangrove species including *C. tagal* (Popp, 1984). However, GC alone was used for the identification of the "pinitol." Since it was not possible to resolve pinitol from MMI using the various chromatographic conditions described in our work, the now positive identification of MMI, by NMR spectrometry, intimates that Popp was mistaken in designating the *O*-methyl derivative as pinitol.

Anomalies in anticipated carbohydrate compositions were observed in the major operationally defined fractions obtained using traditional methods, e.g., α -cellulose contained arabinose and glucose in equal proportions, and contained 7% uronic acids instead of mostly glucose. High levels of uronic acids and arabinose also were found in most of the other fractions isolated in this way. So, although the yield of pectic substances from conventional oxalate extraction was low (4–6%), it was apparent that in total the leaves consisted of unusually high proportions of pectic substances. To circumvent the difficulties in interpreting these compositional data and consistent with a desire to preserve the chemical integrity of the leaf constituents, a simpler approach was chosen that produced extractives, EDTA (or water) -soluble and -insoluble components (Table 3). These fractions were then analyzed in detail (Table 4 and 7). Corresponding *N. smithi* fecal material was also studied using this approach (Tables 5 and 6).

As mangrove leaves contain a high content of (poly)phenolic material, an effort was made to estimate the contribution of flavonoids to the total phenolics in absolute methanol extracts of the major components of the leaves, namely the aqueous acetone extracts and EDTA-insoluble fractions (Table 3). The Folin-Ciocalteu reagent (Price and Butler, 1977) for total phenolics and the vanillin reaction (Burns, 1971; Ribereau-Gayon, 1972b), specific for phloroglucinol ring functionality in condensed tannins were evaluated.

Quantification was precluded by the presence of a colloidal haze in the Folin-Ciocalteu assay solutions of all samples, except intact senescent leaves (S0), which could not be removed by centrifugation (3000g, 10 min). Nevertheless, in qualitative terms, the total phenolics decreased from very high to very low in the extracts from S0 through to S8. The disappearance of these

TABLE 3. FRACTION DISTRIBUTION IN *Ceriops tagal* LEAF SAMPLES

Leaf sample	Fraction (% leaf dry wt)			
	Acetone-soluble ^a	EDTA-soluble	EDTA-insoluble	Dialysate ^b
Green ^c	22.9	15.2	62.9	0.0
Senescent ^c	22.1	14.7	59.3	3.9
S2 ^d	12.3	12.6	67.5	7.6
S4	9.6	5.6	60.2	24.6
S6	9.0	8.8	69.0	13.2
S8	7.4	2.9	61.7	28.0

^aIncludes pigments, free sugars, flavonoids, and salt(s).

^bCalculated by difference and represents water-soluble material <14,000 mol wt.

^cGreen and senescent leaves contain 7.4 and 7.2% sodium chloride, respectively.

^dSenescent leaves subjected to biodegradation for two weeks, etc. . . . (Giddins et al., 1986).

components is due to leaching and to microbial degradation (Cundell et al., 1979; Giddins et al., 1986; Benner et al., 1986). Negligible amounts of the phenolics were found in the insoluble walls of the leaves, but further extraction of the walls with absolute acetone removed material that was soluble in absolute methanol and gave a strong Folin-Ciocalteu reaction with no haze.

Although this approach therefore was not applicable to these extracts, the above observations did reflect the complex nature of the phenolics in the leaves and exemplifies the inherent difficulty in estimating the contribution of condensed tannins to total phenolics in ecological studies (Mole and Waterman, 1987).

The leaching and degradative processes are further confirmed in Table 3 where there was a large reduction in the amount of acetone-soluble (pigments, free sugars, flavonoids, etc.) and polymeric water-soluble material contained in the aging leaf detritus. Without allowing for dry matter disappearance data, the relative proportion of the insoluble fraction which includes the cell walls, remained constant with time. The increased amount of dialyzable material (< 14,000 mol wt) in the older leaves is also indicative of the depolymerization processes occurring during decomposition.

Two capillary columns (BP20 and BP225) were evaluated for neutral sugar analysis. On the BP20 column, several artifact peaks eluted at R_t (retention time relative to internal standard) < 0.488 (vs. rhamnose 0.615), while mannose coeluted with inositol ($R_t = 1.537$), and glucose eluted before galactose (R_t s = 1.636 and 1.676, respectively). The BP225 column, on the other hand, eluted the artifact peaks at $R_t < 0.344$ (vs. rhamnose 0.485) and gave good separation of mannose, galactose, glucose, and inositol (R_t s = 1.122, 1.212, 1.292, and 1.335, respectively).

The carbohydrate data for the insoluble fraction (Table 4) show that green and senescent leaves have about the same amount of neutral sugars, while the senescent leaves have more uronic acids (predominantly galacturonic acid). The leaves have similar quantities of arabinose and glucose, which is unusual for the higher plants, and are rich in uronic acids. It is also worth noting that low yields of glucose indicate an unusually low concentration of cellulose in these mangrove leaves, especially since some of the glucose must be present as starch. This observation was supported by the low yield (ca. 9–11%) of α -cellulose (Table 2). The analyses confirmed that mangrove leaves have a large amount of pectic material. The composition changed dramatically during degradation. After eight weeks, carbohydrates were reduced by 70%, while apparent lignin content doubled, presumably because of the relative resistance of lignin to biodegradation. The apparent lignin material also includes insoluble polymers from the thick waxy cuticle that is characteristic of mangrove leaves. All of the carbohydrate constituents were reduced by biodegradation, but generally there was preferential digestion of pectates, reflected by the large reduction in arabinose

TABLE 4. COMPOSITION (% DRY MATTER) OF INSOLUBLE FRACTION OF *Ceritops tagal* LEAF SAMPLES FROM TABLE 3

Leaf sample	Neutral sugars						Total neutral sugars	Uronic acids ^a	Apparent lignin
	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose			
Green	0.5	7.0	2.0	2.6	1.4	7.6	21.1	6.0	33.2
Senescent	0.6	7.8	2.6	1.4	1.7	6.2	20.2	10.4	25.2
S2	0.5	6.1	3.1	3.2	2.2	7.6	22.7	6.2	47.9
S4	0.3	4.2	1.9	0.9	1.3	4.1	12.6	1.2	50.8
S6	0.5	2.5	1.6	0.7	1.0	3.7	9.9	0.8	51.9
S8	0.4	1.7	1.4	1.0	0.9	3.4	8.7	0.6	49.3

^a Determined by colorimetric assay against D-galacturonic acid standard.

and uronic acids. These data indicate that biodegradation of mangrove leaves introduces 182 g/kg dry matter of total carbohydrates into the environment for utilization as an energy source by microorganisms and higher animals. The unaccounted-for material from the analyses was 39–44% for green and senescent leaves and 23–41% for the degraded leaf samples. Since there is only ca. 3% protein in both types of leaves (Giddins et al., 1986), the discrepancy is most probably explained by the presence of acid-labile polyphenolic material, which has not been investigated further. The problem is associated with the large amount of tannins in mangrove leaves. Residual insoluble tannins have been observed in the neutral detergent fiber residues of other plant species that contain high levels of phenolics (Reed, 1986; McArthur, 1987).

Analysis of the *N. smithi* fecal material from crabs fed on leaf litter (Tables 5 and 6) shows that the crab does not utilize all of the available energy (carbohydrate) but releases into the environment energy in the form of undigested carbohydrates. All dietary constituent sugars were detected in the feces. The insoluble fraction contained a greatly reduced amount (7–11%) of carbohydrate and an enriched amount (27–39%) of apparent lignin. However, unlike the diet material, the fecal matter has reached a constant level of residual carbohydrate and higher lignin contents, indicating that a digestion-resistant fraction containing ca. 13.5% carbohydrate, which includes uronic acids, survives passage through the gut of *N. smithi*. The relative proportions of carbohydrate show that all polysaccharides are degraded to some extent by *N. smithi*'s digestive system and in particular there appears to be some cellulase activity, since the arabinose-to-glucose ratio increases from 0.68 to 0.90.

The composition of the water-soluble fraction in mangrove leaf detritus is given in Table 7. The complex trends in glycoside contents reflect the complex

TABLE 5. FRACTION DISTRIBUTION IN FECAL MATTER PRODUCED BY *Neosarmatium smithi* FED *Ceriops tagal* LEAF DETRITUS (SEE TABLE 3)

Fecal sample	Fraction (% of feces dry wt)			
	Acetone-soluble ^a	EDTA-soluble	EDTA-insoluble	Dialysate ^b
F2 ^c	34.8	23.6	50.6	0.0
F4	12.3	2.3	71.5	13.9
F6	9.4	1.3	79.6	9.7
F8	9.8	2.7	80.9	6.6

^a Unidentified.

^b Calculated by difference and represents water-soluble material < 14000 mol wt.

^c Fecal matter produced when *N. smithi* was fed on litter types S2–S8 (Giddins et al., 1986).

TABLE 6. COMPOSITION (% DRY MATTER) OF INSOLUBLE FRACTION FROM FECAL MATTER PRODUCED BY *Neosartium smithi* FED *Ceritops tagal* LEAF DETRITUS (SEE TABLE 5)

Fecal sample	Neutral sugars						Total neutral sugars	Uronic acids ^a	Apparent lignin
	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose			
F2	0.3	2.3	1.5	0.8	0.9	3.4	9.1	4.4	53.9
F4	0.3	1.8	1.2	0.9	0.9	3.3	8.2	3.9	49.5
F6	0.2	2.3	1.1	0.7	0.8	3.0	8.1	5.7	47.7
F8	0.3	2.5	1.3	0.5	1.1	2.8	8.3	5.1	47.7

^a Determined by colorimetric assay against D-galacturonic acid standard.

TABLE 7. COMPOSITION OF POLYMERIC EDTA-SOLUBLE FRACTION OF *Ceriops tagal* LEAF SAMPLES FROM TABLE 3

Leaf sample	Carbohydrates (mole %)										Dry matter (%)	
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total carbohydrate	Acid-insoluble residue		
Green	2.8	tr ^a	19.9	2.8	3.9	15.0	15.3	40.5	11	9.3		
Senescent	3.8	1.1	22.2	2.8	2.8	18.2	14.7	34.5	16	10.9		
S2	3.3	1.0	15.7	1.9	tr	11.4	6.4	60.3	10	12.4		
S4	5.1	2.0	5.2	2.4	3.9	12.4	18.1	52.0	23	36.6		
S6	5.0	2.4	8.3	2.7	2.5	8.6	10.0	60.6	17	17.2		
S8	tr	tr	4.0	tr	tr	3.4	3.4	89.1	5	13.4		

^aTrace.

chemical nature of this material and of the degradative processes. The large quantity of acid-insoluble residue (phlobaphenes) and the formation of pink anthocyanidins in the acid hydrolysates is indicative of the high proportion of proanthocyanidin material in this fraction (Ribereau-Gayon, 1972c). The amount of anthocyanidins was highest in the extracts from green leaves and declined rapidly to be just detectable in S4 leaves. The neutral monosaccharide composition of the water-soluble fraction after hydrolysis was similar to that of the insoluble fraction, with arabinose, galactose, and glucose being the major sugars, while rhamnose, fucose, xylose, and mannose were the minor ones. The polysaccharide component in these fractions contained 35–89% uronic acids. These results confirm that pectates are complexed with or bonded to the proanthocyanidin. Poor recoveries were obtained from the analysis of these water-soluble fractions, and once again this loss could only be explained by the presence of a large amount of polyphenolic material that is acid-labile. In this case, agreement was obtained between GC and corrected phenol-sulfuric acid values for the carbohydrate measurements. However, it should be noted that due to interference by flavonoids, significant errors may result when the carbohydrate contents in flavonoid-rich materials are determined by phenol-sulfuric acid analysis (Rahman and Richards, 1987). A corresponding soluble fraction from *Rhizophora stylosa* was designated flavoglycan (Neilson et al., 1986a) during the preliminary investigation of the diagenesis of mangrove leaves, implying covalent linkages between flavonoid and polysaccharide components. One of us (G.N.R.) has subsequently found evidence that condensed tannin-polysaccharide complexes involve strong noncovalent interpolymer bonding (Rahman and Richards, 1988), and further studies are continuing on these complexes.

The leachate from mangrove leaves introduces a large amount of organic material into the mangrove environment. If the tannin concentrations in the soluble fraction are high, then the leachate is inhibitory to the uptake by organisms (Benner et al., 1986; Alongi, 1987), and this inhibition will extend to any carbohydrate material that may be bound to the tannins. Furthermore, leachable tannins have been demonstrated to significantly reduce consumption rates of mangrove leaves by *N. smithi* (Neilson et al., 1986b) prior to leaching. Nevertheless, Benner et al. (1986) have shown that 42% of some leachates can be utilized by microorganisms and are therefore available to animals in the estuarine food web.

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FUNCTION OF GLANDULAR SECRETIONS IN FRAGRANCE COLLECTION BY MALE EUGLOSSINE BEES (Apidae: Euglossini)

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Abstract—Male *Eulaema cingulata* (Fabricius) (Apidae: Euglossini) possess large cephalic labial glands that secrete a mixture of lipids. In the process of fragrance collection, males secrete the labial gland lipids onto the substrate. The mixture of lipids and fragrances is then taken up by the front tarsal brushes and transferred to the hind tibial organs. The labial gland secretions apparently serve as a nonpolar solvent and carrier that increases the efficiency of fragrance collection.

Key Words—Euglossini, *Eulaema cingulata*, Hymenoptera, Apidae, labial gland, lipid, fragrance, orchid, tibia.

INTRODUCTION

Euglossine bees (Apidae: Euglossini) are conspicuous members of the neotropical bee fauna. Male euglossine bees are noted for their unique behavior of collecting fragrances from flowers of orchids, aroids, and other plants and from rotting wood and fruits. The male bees possess numerous morphological specializations related to fragrance collection. Their front tarsi are modified into moplike brushes and their hind tibiae possess a deeply invaginated, hair-filled cavity. The male bees brush the surface of a flower or other source with the front tarsal brushes and then transfer the fragrance compounds (mostly mono- and sesquiterpenes and aromatics) to the hind tibial organs. We still do not know what the male bees do with the collected fragrances. Dressler (1982)

reviewed the biology and fragrance-collecting behavior of male euglossines; additional relevant works include Kimsey (1980, 1984), Schemske and Lande (1984), and Ackerman (1983).

Earlier investigation of male euglossine exocrine glands (Williams and Whitten, 1983) showed that the chemical contents of the cephalic glands and the hind tibial organs are similar; both contain a mixture of normal acetates, alcohols, alkanes, alkenes, and other lipids. Careful dissection and analyses have revealed that these cephalic lipids are produced by the labial glands, and not by the mandibular glands as we reported earlier. The hind tibial organ may also contain a variety of fragrance compounds including mono- and sesquiterpenes and simple aromatics.

The morphology of the hind tibial organ suggests that it may be more than a passive storage area for fragrances. The structure of the male hind tibia was summarized by Dressler (1982), and the ultrastructure of the tibial organ was studied by Cruz-Landim et al. (1965). The anterior surface of the hind tibia bears an elongate sunken pit ("scar") filled with long hairs. The collected fragrances are placed on these hairs and are drawn into the interior of the tibia by capillary action. A narrow canal connects the scar with the interior of the organ. The interior is filled with tangled, branched hairs that form a spongelike structure. Cruz-Landim et al. (1965) describe two distinguishable layers of glandlike tissue lining the organ. The presence of pinocytotic vesicles, microvilli, and numerous mitochondria suggest that the tissue is metabolically active, possibly involved in secretion or uptake of compounds and that it is not just a passive storage organ.

The presence of the same set of lipids in both the labial glands and the hind tibia is puzzling. Earlier, we hypothesized that the labial secretions might serve as pheromones, similar to their role in bumble bees (Williams and Whitten, 1983). We now present evidence that the male bees secrete large quantities of labial gland lipids onto fragrance-laden substrates, mop up the mixture with their front tarsal brushes, and then transfer the mixture to the hind tibial organs. These secretions probably serve as a nonpolar solvent to increase the efficiency of fragrance collection.

METHODS AND MATERIALS

In the present study, we posed the following questions: (1) What hexane-extractable lipids occur in the labial glands and hind tibial organs of male *Eulaema cingulata*? (2) Do the male bees secrete lipids onto the substrate from which they are collecting fragrances?

We studied the fragrance-collecting behavior of bees at two types of fragrance-bearing substrates: one artificial and the other naturally occurring. The

first consisted of squares of filter paper baited with skatole, a fragrance chemical known to attract *Eulaema cingulata*. The natural substrate consisted of wounded bark and wood of a leguminous tree, *Dalbergia cubilquitensis* Pittier, used for shading cacao at the study site. For several years, we have observed male *Eulaema cingulata* brushing on discolored trunk wounds of several of these trees. The wounds are mildly fragrant, and the bees are probably attracted by sesquiterpenes produced by the fungus-infected wound.

Field work was conducted during December 1986 at Estación Experimental La Lola near Siquirres, Limón Province, Costa Rica. Although the vegetation consists of managed cacao groves, the area supports high populations of several euglossine species. Samples (hexane extracts of plant or bee parts or of filter paper) were returned to Florida and analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett-Packard 5995B with a 30-m methyl silicone capillary column. Oven temperature was programmed from 40 to 290°C at 8°C/min; injection port temperature was 300°C. Most compounds were identified by comparison with authentic standards, and spectra of unidentified compounds were searched against the NBS mass spectral library.

Bee Gland Contents. Male *E. cingulata* were attracted to 4 × 4-cm blotter pads saturated with skatole. Eight bees were collected and the heads and hind tibiae extracted separately in vials containing 1.0 ml hexane. Samples were analyzed by GC-MS.

Lipid Secretion Experiment. To determine whether the male bees secrete lipids onto a fragrance-laden substrate during brushing activity, we allowed bees to brush on a filter paper substrate suspended over a fragrance source (blotter paper saturated with skatole). A 4-cm² hole was cut in the top of a plastic petri dish. A 9-cm round piece of filter paper was perforated with 5–10 holes using a paper punch to facilitate diffusion of skatole vapors. The paper was then secured to the inner surface of the cover. A small pad of blotter paper saturated with skatole was attached to the inside of the Petri dish bottom. The assembled dish allowed the skatole vapor to diffuse through the holes in the paper. Bees could brush on the surface of the paper without contacting the concentrated skatole below. Subsequently, the filter paper could be extracted and analyzed without the interfering presence of large amounts of skatole. Skatole-baited dishes were distributed in each of two habitats at La Lola during February 27–March 2. Initially, two dishes were suspended in cacao trees in the relatively open *Dalbergia* site and another two in the densely shaded Barker cacao site. For each of two subsequent days, three dishes were placed in each of these habitats. In order to estimate the number of bees visiting each paper, the number of bees present at each paper was recorded at two 2-hr intervals throughout the day. After 12 hr, the filter papers were removed and extracted with 2 ml of hexane. The extracts were analyzed by GC-MS.

Fragrance Accessibility. In order to test the hypothesis that bees might

deposit greater quantities of lipids on "dry" substrates that are not saturated with fragrances than on substrates that are saturated with fragrance compounds, we set out six skatole-baited Petri dishes in the Barker cacao site for 12 hr. Three of these dishes had the fragrance inaccessible on the blotter paper, as described above, whereas in the other three dishes the skatole was applied directly to the surface of the filter paper where bees could have direct contact with it. Collection and extraction of the lipids deposited on the filter paper were conducted as described above for the lipid secretion experiment.

Observations of Bee Behavior. We observed the behavior of bees at the skatole-baited Petri dishes to determine whether or not bees utilize their mouthparts, legs, and other structures in the deposition of lipids on substrate surfaces. While engaged in brushing behavior, the bees are much less wary than normal and can be approached slowly and observed at close range (0.5 m or less).

Dalbergia Tree Samples. To determine the distribution of compounds within the tree, samples of twigs, leaves, bark, and wood were collected and extracted in hexane. A tree corer was used to sample tree wounds. The cores were cut into 3-mm segments, and the segments were extracted separately in 1 ml of hexane. Samples were analyzed by GC-MS.

Bioassays. Crude field bioassays were conducted with several of the major compounds of the labial secretions of *Eulaema cingulata*, with extracts of *E. cingulata* heads, and with samples of *Dalbergia* wood. The compounds tested were synthetic tetradecyl, hexadecyl, and octadecyl acetates and all possible combinations of these compounds. One drop of the pure compound (or 1:1 v/v mixture) was applied to a blotter pad tacked at breast height on cacao trunks. Individual hexane extracts of male *E. cingulata* heads were applied to blotter pads ($N = 5$). All samples were monitored for bee activity for 5 min every hour from 800 to 1800 hr for one day. Core samples were taken from healthy, undamaged *Dalbergia* trunks ($N = 3$) and also from discolored tree wounds ($N = 3$).

RESULTS

Bee Organs. The major compounds present in the labial glands of male *E. cingulata* are tetradecyl, hexadecyl, and dodecyl acetates (Table 1). A series of normal alkanes and alkenes ranging from C_{25} to C_{33} is also present. The rank order of abundance of these compounds is highly consistent among individuals. The total quantity of labial gland lipids is more variable, ranging from 0.2 to 5.9 mg (mean = 4.6 mg).

The hind tibial organs contain the same set of compounds that is present in the labial glands, plus several other classes of compounds (Table 1). A wide variety of monoterpenes, sesquiterpenes, and aromatics may be present. These

TABLE 1. MEAN PERCENT COMPOSITION OF HEXANE EXTRACTS OF HEADS AND HIND TIBIA OF *Eulaema cingulata* AND *Dalbergia cubilquitenzis* TRUNK WOUNDS

Compound	Head (N = 8)	Wood (N = 1)	Legs (N = 8)
Benzaldehyde	0.0	0.0	2.6
β -Ocimene	0.0	0.0	1.3
<i>trans</i> -Limonene oxide	0.0	0.0	0.3
Benzyl acetate	0.0	0.0	0.1
Dihydrocarvone	0.0	0.0	0.4
Carvone	0.0	0.0	0.3
Anisaldehyde isomers	0.0	0.0	0.4
<i>trans</i> -Carvone oxide	0.0	0.0	9.7
Phenylethyl alcohol	0.0	0.0	4.5
<i>p</i> -Dimethoxybenzene	0.0	0.0	0.6
Phenylethyl acetate	0.0	0.0	1.4
Indole	0.0	0.0	1.2
Skatole	0.0	0.0	0.3
Eugenol	0.0	0.0	0.1
Unidentified sesquiterpenes	0.0	0.8	0.0
Dodecene	0.4	0.0	0.0
Dodecane	0.2	0.0	0.0
Dodecyl acetate	5.8	1.3	0.0
Tetradecene	1.7	2.1	0.1
Tetradecane	1.2	0.0	0.0
Tetradecyl acetate	67.5	39.6	4.0
Hexadecene	1.3	0.7	1.0
Hexadecyl acetate	8.3	14.9	0.2
Unidentified	0.0	0.0	1.3
Octadecenyl acetate	1.5	1.9	0.0
Octadecyl acetate	1.2	3.6	0.3
Pentacosene	0.0	1.3	0.0
Pentacosane	0.9	3.1	3.1
Unidentified	0.0	0.0	0.2
Heptacosene	2.8	12.1	22.7
Heptacosane	0.1	1.0	1.2
Nonacosene	0.8	4.9	5.0
Unidentified	1.1	3.4	0.7
Hentriacontene	0.1	0.0	0.2
Hentriacontane	0.0	0.0	0.7
Dodecyl octadecenoate	0.6	0.0	0.0
Tetradecyl octadecenoate	3.7	0.0	6.8
Hexadecyl octadecenoate	0.5	0.0	6.1
Unidentified waxy ester 1	0.0	0.0	2.9
Unidentified waxy ester 2	0.0	0.0	1.9
Unidentified waxy ester 3	0.0	0.0	0.8
Unidentified waxy ester 4	0.2	0.0	5.0
Unidentified waxy ester 5	0.0	0.0	1.2
Octadecyl octadecenoate	0.1	0.0	5.6

TABLE 1. Continued

Compound	Head (N = 8)	Wood (N = 1)	Legs (N = 8)
Unidentified waxy ester 6	0.0	0.0	1.7
Unidentified waxy ester 7	0.0	0.0	2.7
Unidentified waxy ester 8	0.0	0.0	0.3
Unidentified waxy ester 9	0.0	0.0	0.8
Unidentified waxy ester 10	0.0	0.0	0.2
Eicosanyl octadecenoate	0.0	0.0	0.2
Unidentified base peak = 256	0.0	0.7	0.0
Unidentified base peak = 254	0.0	4.7	0.0
Unidentified base peak = 300	0.0	0.5	0.0
Unidentified base peak = 300	0.0	1.8	0.0

compounds give the tibial extracts a perfumelike odor. The kind, number, and quantity of these fragrance compounds varies greatly among individual bees; this variation presumably reflects the varied sources (flowers, fungi, etc.) from which the compounds are collected. A second class of compounds in the tibial organs is tentatively identified as high molecular weight waxy esters. One homologous series consists of tetradecyl, hexadecyl, and octadecyl octadecenoates. The quantities of these waxy esters vary greatly among individual bees, and it is uncertain whether they are collected from floral and/or fungal sources or whether they are secreted by the bees. Some of these waxy esters may be present in the labial glands in small quantities.

Lipid Secretion Experiment. Most of the papers were heavily visited by fragrance-collecting male *E. cingulata* and *Euglossa dodsoni* Moure during the morning and early afternoon. After 12 hr, the exposed area of the filter paper was translucent, as though grease had been applied to the paper. The hexane extracts of the papers (Table 2) contained large quantities of the same lipids present in the labial glands of *E. cingulata*. Representative chromatograms of extracts of labial gland, hind tibia, and papers are shown in Figure 1. Also present were smaller amounts of the lipids present in the labial glands of *Euglossa dodsoni*, such as eicosenyl-1,20-diacetate and eicosenol. The quantity of total lipids deposited on the filter papers ranged from 2 to 55 mg (mean = 16.5 mg). The largest quantity represents about 12 glandular equivalents of *Eulaema* labial secretions deposited in 12 hr.

Dalbergia Bark Wounds. The wounds in the *Dalbergia* bark that attract *E. cingulata* are either natural, caused by insect damage, or are man-made wounds such as machete cuts. The natural wounds appeared as small holes (1–2 cm diameter) or fissures in the bark on either the main trunk at heights up to 5 meters or on small stump sprouts at the base of the tree. Fresh machete wounds

TABLE 2. COMPOSITION OF HEXANE EXTRACT OF FILTER PAPER SUBSTRATE

Compound	Amount	
	Percent	Milligrams
Skatole	1.9	0.3
Dodecyl acetate	2.1	0.3
Tetradecyl acetate	44.9	7.4
Hexadecyl acetate	30.5	5.0
Octadecenyl acetate	11.7	1.9
Octadecyl acetate	1.3	0.2
Pentacosane	1.7	0.2
Heptacosene	0.6	0.1
Other compounds	6.3	1.0
Total	100.0	16.5

did not attract bees, but bees were observed at machete wounds that were 3–6 months old. The wounds visited by bees did not produce a liquid exudate, but the surface of the exposed wood was colored dark brown.

We observed male bees collecting fragrances from the wounds during vis-

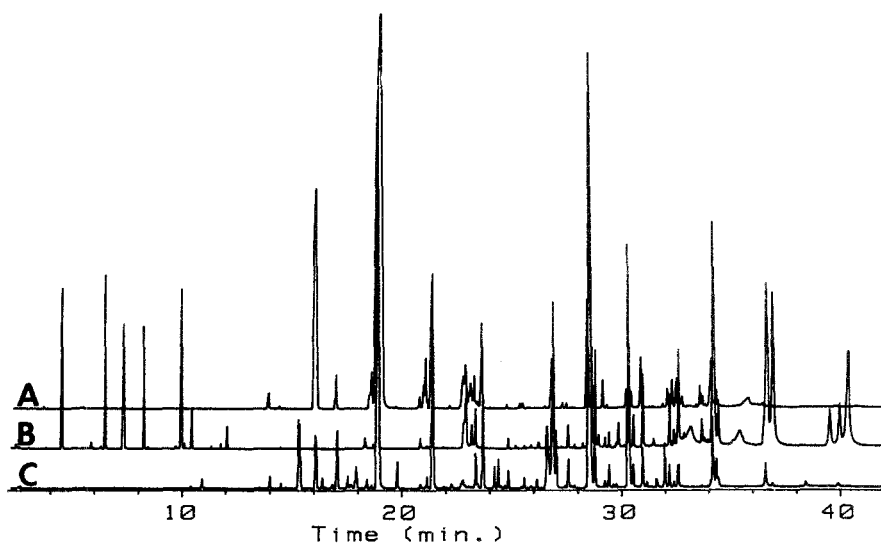


FIG. 1. Representative total ion chromatograms of hexane extracts of *Eulaema cingulata* labial gland (A), hind tibia (B), and filter paper substrate (C) on which the bees had brushed. Note the qualitative similarity of the three chromatograms, except for the fragrance compounds present in the hind tibia (peaks from 4 to 12 min.).

its to La Lola over several years. Bees were seen at the wounds during November 1984, March and September 1985, and July and December 1986. No bees were seen during February 21–24, 1986. Most fragrance-collecting activity occurred between 800 and 1600 hr.

Analyses of extracts of the core samples from the tree wounds revealed three classes of compounds (Table 1). The first consists of several unidentified sesquiterpenes. The major sesquiterpene also occurs in the fragrance of the euglossine-pollinated orchid *Polycynis gratiosa* (Whitten, unpublished); therefore, it is likely that this compound is attractive to some euglossines. The second class of compounds includes all the major lipids found in the labial gland extracts of *Eulaema cingulata*. The third class of compounds consists of a group of unidentified compounds with distinctive mass spectra in which the base peak is also the apparent molecular ion. These compounds do not occur in the bee glands or in the fragrance of any orchid we have analyzed. Analyses of 3-mm segments of the core revealed that the lipids are present only in the surface layers of the wood (the outer 6 mm). The sesquiterpenes and the unidentified compounds are present deeper into the wood, including the heartwood (36–42 mm deep); the areas that contain these compounds are colored dark reddish brown and produce a pleasant aroma. The healthy, unpigmented wood from 12 to 33 mm deep contained no hexane-extractable compounds and was not fragrant.

Microscopic examination of wood sections from the tree wounds stained with lactophenol cotton blue revealed fungal hyphae; one sample from the pith of a wounded stump sprout revealed fruiting bodies suggestive of *Fusarium*. Fungal hyphae could not be detected in the highly discolored wood and heartwood because the ergastic substances masked the stain.

Bioassays. The field bioassays using the *Dalbergia* healthy bark and wood and the *Eulaema* cephalic extracts did not attract bees, but core samples of the tree wounds began attracting male *E. cingulata* within minutes of removal from the tree. Both the surface layer of the wound and also the deeper heartwood were fragrant and attracted male bees ($N = 4$, surface; $N = 6$, heartwood).

Bee Behavior. Although fragrance-collecting bees can be observed at close range, the rapidity of movements makes observations of behavior difficult. On all substrates, the bees land on the substrate and usually touch the substrate rapidly with their half-open mandibles. The substrate is then brushed intensely with the front tarsal brushes, with the antennae touching the same area of the substrate. Brushing may continue for up to $1\frac{1}{2}$ min. The bees then hover and transfer the collected material from the front tarsi to the basitarsal comb of the midleg, and then to the slit of the hind tibial organ. This behavior has been described in detail by Kimsey (1984). However, the bees occasionally wipe the mouthparts with the front tarsal brushes during hovering and transfer behavior. Our observations suggest that labial secretions might be applied to the substrate

either directly via the mouthparts or indirectly via the front tarsal brushes. More careful observations with high speed cinematography are necessary to determine how the lipids are applied to the substrate.

DISCUSSION

Male euglossines possess large cephalic labial glands that produce copious amounts of various lipids. The hind tibial organs also contain these lipids, together with a variety of fragrant compounds presumably collected from flowers or fungus-infected wood. The labial gland secretions of male euglossines are chemically similar to those of *Bombus* and other apid bees (see review by Duffield et al., 1984). Male *Bombus* appear to use their labial gland secretions to mark territories and to attract females (Kullenberg et al., 1970, 1973). There is no evidence that euglossines use their labial secretions in a similar manner (Kimsey, 1980), and we know of no reports of marking behavior in euglossines.

It is possible that the lipids are secreted by the hind tibial organs or by the front tarsi, but the size and contents of the labial glands makes them the most probable source. We presume that the lipid secretions are also applied to the surface of orchids and other flowers visited by fragrance-collecting male euglossines. Unfortunately, none of the euglossine-pollinated orchids (e.g., *Catasetum*) were in flower at the field site during our study. The accessible vs. inaccessible fragrance experiment showed that the bees applied large amounts of lipids to the substrate in both cases.

The *Dalbergia* wounds that attract male *E. cingulata* contain a large number of chemicals whose origin and attractiveness to the bees is uncertain. The data presented above suggest (but do not prove) that the bees are attracted to the wounds by volatile sesquiterpenes produced by microorganisms in the wound or produced by the infected wood. The lipids are present only in the surface layers of the infected wood. This superficial distribution is consistent with the hypothesis that the bees secrete these lipids onto the wood and that the lipids are not secreted by the tree or by fungi.

We hypothesize that these lipids are used by the bee to increase the efficiency of fragrance collection by serving as a nonvolatile, nonpolar solvent to dissolve and retain the fragrance compounds. In the process of brushing and the transfer of fragrances to the hind tibiae, there might be considerable loss of highly volatile monoterpenes due to the rapid air currents around the hovering bee. Also, the fragrances might evaporate from the surface of the hind tibial slit before being drawn into the inner cavity of the tibia. By mixing the fragrances with an oily carrier such as the labial secretions, the losses due to evaporation should be greatly reduced, and the efficiency of fragrance collection might be greatly improved. The use of lipids to dissolve and retain flower fra-

grances has long been used by the perfume industry in the "enflourage" technique. In this method, flowers are laid upon sheets of glass that are coated with a thin layer of grease. The volatile fragrances emitted by the flowers dissolve into the grease, and the grease is removed and extracted to produce a floral essence. It appears that euglossine bees use lipids in a similar manner to collect and retain floral "perfumes."

The data presented above suggest that fragrance-collecting euglossines might accumulate large amounts of labial gland lipids in the hind tibial organs. Since the bees may live and collect fragrances for several months, some mechanism must exist for removing lipids from the tibial organ; otherwise, the organ would quickly fill with nonvolatile lipids. We speculate that the labial lipids are taken up selectively across the highly invaginated inner lining of the tibial organ, leaving the fragrance compounds in the tibial cavity, and that the lipids are either catabolized or are translocated back to the labial gland for reuse. The interior surface of the tibial organ, with its high surface area and metabolically active lining, appears well-adapted for the uptake of chemicals from the cavity. The transport of the lipids might occur via a lipophorin transport system, as described in other insects by Chino and Downer (1982). Tracer experiments indicate that deuterated hexadecyl acetate is rapidly translocated from the hind tibiae to the labial gland (Whitten and Williams, unpublished).

If such translocation and recycling of labial gland lipids does occur, then the glandular and fragrance collecting apparatus of male euglossines can be regarded as a sort of chemical conveyor belt. The labial gland secretions are applied to the substrate and carry the fragrances into the hind tibial organ, and then the lipids might be recycled to the labial gland for reuse. Such recycling of lipids might be adaptive in two ways. First, it removes lipids from the hind tibial cavity, thereby preventing it from filling with lipids and allowing further uptake of fragrances. Second, it conserves an energetically expensive exocrine product.

This study does not address what the bees do with the collected fragrance compounds; tracer experiments with labeled fragrances have not been performed. At present, there is no evidence that the fragrance compounds are metabolized or are translocated from the hind tibial organ. We hypothesize that the male bees accumulate large quantities and varieties of fragrances in their tibia and that the fragrances are somehow used in the courtship of female bees. The fragrances contained in the hind tibia can volatilize from the organ, and live, captive bees are fragrant to the human nose (and presumably to other bees). The tibial "bouquet" might serve as an indicator of male fitness to females.

The role of euglossine labial secretions in fragrance collection represents a novel use of labial secretions among bees. However, fragrance-collecting behavior has also been reported from anthophorid bees (Norden and Batra,

1985). Male *Centris* chew the stems of parsnip plants and absorb the fragrant juices in specialized labral "moustaches." The bees then mark objects in their territories by rubbing the objects with the moustaches. It would be interesting to analyze the applied juices to determine whether *Centris* might also use labial gland secretions as a carrier for plant volatiles.

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QUANTITATIVE BIOASSAY FOR CHEMOTAXIS WITH PLANT PARASITIC NEMATODES

Attractant and Repellent Fractions for *Meloidogyne incognita* from Cucumber Roots

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Abstract—A simple, flexible, and quantifiable bioassay for the attraction or repulsion of plant parasitic nematodes to or from root fractions or pure substances is described. Accurate gradients of volatile and nonvolatile substances can be measured and established. The method entails placing the nematodes in narrow agarose tracks such that their movement is essentially linear and the distance a population has traveled away or toward a given substance can be monitored with time. Plastic plates, each containing 10 such tracks, are described. The method is illustrated with second-stage larvae of *Meloidogyne incognita* and a volatile attractant and nonvolatile repellent fraction obtained from cucumber roots.

Key Words—Chemotaxis, bioassay, nematodes, *Meloidogyne incognita*, attractants, repellents, cucumber roots, gradients.

INTRODUCTION

In 1925, Steiner proposed that plant parasitic nematodes located their hosts by chemoreception (Steiner, 1925). In support of this view, it is now well established that nematodes accumulate about the roots of host plants (Lownsbery and Viglierchio, 1960, 1961; Azmi and Jairajpuri, 1977; Prot, 1980; Prot and Van Gundy, 1981). Moreover, a range of plant parasites including *Heterodera schachtii* and *Meloidogyne hapla* (Viglierchio, 1961), *M. javanica* (Riddle and Bird, 1985), *Aphelenchoides besseyi* (Lee and Evans, 1973), *Hirschmanniella*

oryzae (Bilgrami et al., 1985), and others (Green, 1971) has been demonstrated to be attracted or repelled by plant roots and their emissions. Furthermore, a leaf attractant for *Orrina phyllobia* has been extracted from *Solanum elaeagnifolium* (Robinson and Saldana, 1988). However, none of the substances responsible for any of these interactions has been isolated or identified. While knowledge of the character of root emissions has increased (Schwab and Leonard, 1984; Thompson, 1985), no specific set or subset of these compounds has been delineated that elicits chemotaxis from nematode parasites.

On the other hand, β -myrcene has been found to be a potent attractant for the pine wood nematode *Bursaphelenchus xylophilus* (Ishikawa et al., 1986). In addition, simple inorganic salts of Na^+ , Mg^+ , Cl^- , and OAc^- have been reported recently to attract *Rotylenchulus reniformis* (Riddle and Bird, 1985). The polar structures AMP and cyclic AMP were also attractive to this species, as were germinated host plant seeds. In contrast, *M. javanica* was only attracted to tomato seeds. An apparently general attraction to carbon dioxide (Klinger, 1965; Pline and Dusenbery, 1987) and oxygen (Dusenbery, 1983) has been noted. The response of male *Heterodera glycines* to a variety of substances has been examined (Huettel and Jaffe, 1987). The literature (with reference to the free-living nematode *Caenorhabditis elegans*) has been summarized (Dusenbery, 1983; Ward, 1973). Potential mechanisms for host or prey recognition have been reviewed (Dusenbery, 1983; Zuckerman and Jansson, 1984), and host or prey lectin-nematode carbohydrate interaction has been proposed as a general basis of recognition. The subject of recognition in plant diseases (primarily fungal) has also been generally reviewed (Daly, 1984).

Apart from plant roots, certain nematophagous fungi are known to attract nematodes (Zuckerman and Jansson, 1984; Balan et al., 1976; Klink et al., 1970; Jansson and Norderling-Hertz, 1983). Infection occurs specifically at the chemosensory organ (Jansson and Norderling-Hertz, 1983). Again, no structures have been forthcoming, but a lectin-carbohydrate interaction is suspected (Zuckerman and Jansson, 1984). It should be noted that root exudates can also be repellent (Diez and Dusenbery, 1986) and, in addition, have long been known to induce hatching of certain nematodes (Balam et al., 1949; Carroll, 1958; Hartnell et al., 1960; Jatala et al., 1977; Turner and Stone, 1981; Tanda, 1985). The structure of one such hatching entity, glycinoecleptin, has been characterized recently (Fukuzawa et al., 1985). A variety of simple chemicals can also produce this effect (Clarke and Shepherd, 1966; Jantzen, 1968; Okada, 1972; Greet, 1974; Clarke and Hennesey, 1983). Molting can also be induced by carnation root diffusates (Rhoades and Linford, 1959) and other substances (Shepherd and Clarke, 1971). The potential importance of these various chemosensory processes to nematode control has been summarized by Dusenbery (1987).

Several methods have been employed to assess the attractiveness of materials to nematodes. These range from photographing the tracks (Riddle and Bird,

1985; Ward, 1973) of the animals placed at the perimeter of an agar Petri plate as they move or not to the center (where the attractant is placed) to a video monitoring of all movements with time (Pline and Dusenbery, 1987; Dusenbery, 1983). Simply counting the nematodes in zones of a Petri plate at various distances from the root fragment has also been employed (Bilgrami et al., 1985), as has the aggregation of nematodes under paper disks placed upon such plates (Robinson and Saldana, 1988).

Our bioassay is designed around the concepts of simplicity and effective linear movement of the nematodes. We wanted: (1) the ability to quantitate both nematode movement and chemical gradient; (2) reproducibility; (3) flexibility of individual experimental design (e.g., competition between two attractants, a variety of concentrations, etc.); (4) the capacity to use very small amounts of substance as attractant or repellent; (5) a design that would not lose volatiles, and (6) to give the nematodes equal opportunity to move away from or toward a substance or fraction. In this work we describe such an assay and illustrate its effectiveness with attractant and repellent fractions obtained from cucumber roots for second-stage larvae of the plant parasite *Meloidogyne incognita*.

METHODS AND MATERIALS

¹⁴C-Labelled glycerine, glycine, and *n*-propanol were commercial samples (New England Nuclear) and used without purification. Agarose was obtained from International Biotechnology, Inc.

Second-stage larvae of *M. incognita* (MiL₂) were obtained from female egg masses isolated from tomato roots. Cut roots were blended in 3% commercial bleach. Eggs were washed through 40- and 170-mesh screens and collected on a 500-mesh sieve and washed. They were placed on a filter paper above a deep Petri plate. Hatching was best at 26°C. After collection and storage at 15°C, the settled larvae are concentrated by decanting.

Cucumber root exudates and fractions were obtained by three procedures. The first consisted of immersing the roots of young plants in tap water and aerating them for one day. In the second, larger batches of plants grown in vermiculite were leached once after four days and resupplied with fresh water. After 24 hr, the water from the root matrix was withdrawn. The third procedure, similar to the second, entailed holding the plants in a large pan in a sealed glove bag. Gases and volatiles from the aeration were passed through a series of traps.

Aqueous exudate fractions (6 liters) were filtered and concentrated on a rotary evaporator at 45°C and water pump pressure of ~20 mm. The dry concentrate was either extracted directly with cold 95% ethanol or triturated with 10 ml of water and extracted with methylene chloride. The methylene chloride extract was stripped on the rotary evaporator and dissolved in 1 ml of 95%

ethanol. The distillate and constituents of the ice or CO₂ trap preceding the water pump were also extracted with methylene chloride, concentrated, and taken up in ethanol. Note: 1 μ l of neat ethanol used in the bioassay procedure (see below) had no effect upon the nematodes.

Bioassay. We have designed a block and lid bioassay device from Lucite (polymethyl methacrylate), transparent plastic blocks that contain 10 parallel tracks, 90° V grooves 2 mm deep and 4 mm across the top. Each groove is 7.5 cm long. The block is sealed at each end. Corners are rounded. Overall outer dimensions are 9 × 8.5 × 1.3 cm. All faces are polished and flat. In addition, each block is fitted with a transparent gasket and lid; the lid has the same outer dimensions as the block but is thinner (0.5 cm). The lid is manufactured such that when screwed to the bottom block (a thumb screw at each corner) ten 1-mm holes in it are aligned exactly with the center of each track. The thin flexible gasket is lined at right angles to the tracks from the center out every 0.5 cm. A small center section is cut from the gasket to allow access to the tracks from the holes in the lid. The gaskets were cut from a polyester "plain paper copier transparency film" (Minnesota Western). The lines were copied onto the gasket sheet with a photocopying machine. One of the "race blocks" is sketched close to actual size in (Figure 1). (Appropriately placed holes in the gasket can replace the center cutout. This latter form of gasket is more appropriate for testing volatile substances.) The unit is arranged such that examination of the plates with a dissecting scope allows one to see the grid marks (gasket) and the nematodes along the various sections of each track.

For maximum nematode mobility and visibility, each groove is supplied with 150 μ l of warm 0.8% agarose. Thus, each 0.5-cm section of the 7.5-cm track contains 10 μ l of matrix. In this way, the level of the matrix in each groove is about $\frac{2}{3}$ up from the bottom. This allows for a layer of air over each entire track length. Substances or root fractions are injected or not into the end of each agarose track via hypodermic syringe. The gasket and lid are fastened in place. After a suitable time, the nematodes are injected in aqueous suspension into the center of each track via the alignment holes; usually 50–100 animals in 1 μ l are placed in each track. Because of condensation, counts are best made with the tracks upside down. Counting nematodes in a track requires 5–10 min; events are recorded at specific times as the number of nematodes in each section. Several concentrations and controls can be run on the same block in each experiment.

Gradients. Concentration time gradients for glycerine, glycine, and *n*-propanol in the tracks were determined using ¹⁴C-labeled substances. Thus, 1 μ l of an aqueous solution of substrate is injected into the end of each track such that the end 0.5-cm section will be at the desired concentration (e.g., 10⁻³ M). At intervals, tracks were sectioned at each 0.5 cm and each section was dis-

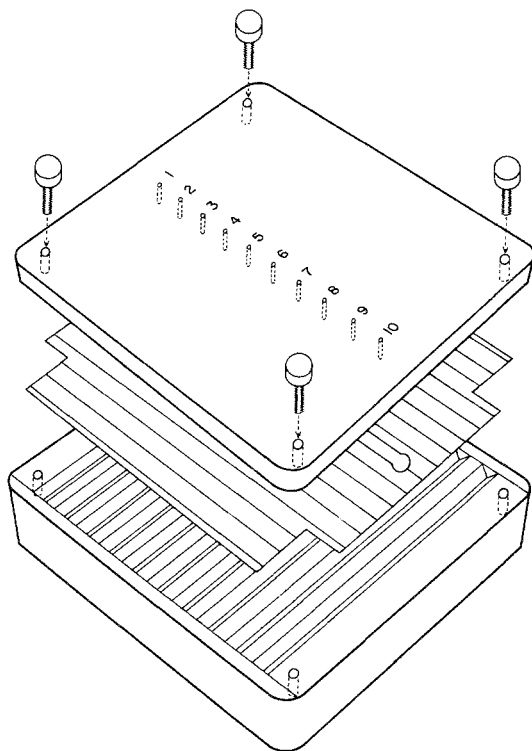


FIG. 1. Assay block showing grooves, gasket, and lid ($\frac{3}{4}$ actual size); reduced 25% for reproduction.

persed in Packard Insta-Gel Counting Solution and counted with a Packard Tri-carb model 3255 liquid scintillation counter with automatic external standard. Reproducibility of gradient concentrations in each section was $\pm 5\%$. Several gradients are shown in Figure 2.

Quantifying the Assay. While the graphs of nematode population with time (Figures 3-5) allow a qualitative assessment of attraction or repulsion by visual examination, these data can be more simply quantified. We define an attractant-repellent ratio at time t as $(A/R)_t$. This is equal to the ratio of the population densities weighted for the distance they have moved towards or away from a given substance or fraction. Thus, for any track or for the average counts from a number of replicate tracks summation $A = \Sigma(N)(D)$ where N = the number of nematodes in each 0.5-cm section and D is the distance of the center of the section from the middle starting point. For our tracks the fixed D values are

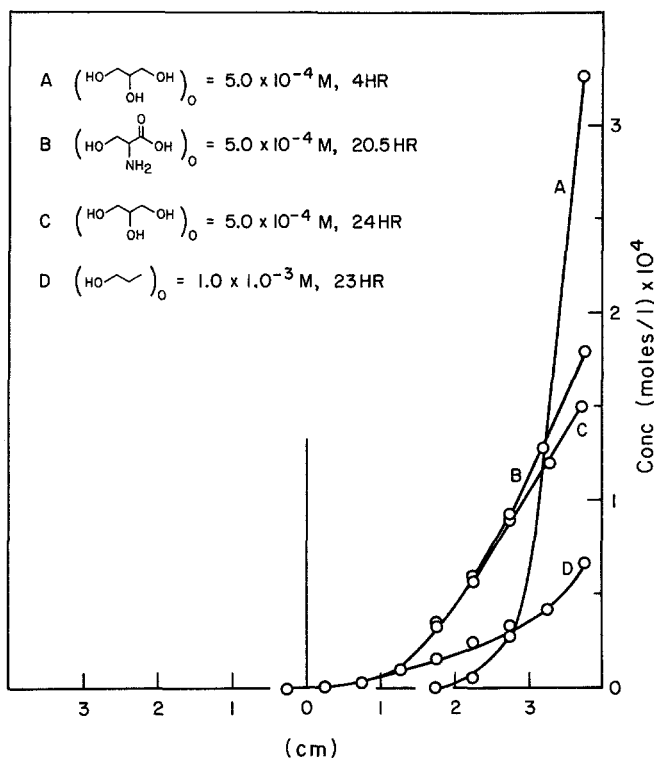


FIG. 2. Gradients of glycerine, glycine, and *n*-propanol.

0.25, 0.75, 1.25, 1.75, 2.25, 3.25, and 3.5 cm. The units for *A* are nematode-centimeters (*n*-cm). A similar expression represents *R*. In this case the counts are taken from the center towards the end of the plate away from the side end into which the test fraction is placed. In our work we have arbitrarily chosen to add the substrate to the end section at the right side of the plate. Thus, in the figures, movement to the right represents attraction and movement to the left represents repulsion. Finally, for control runs, the *A/R* ratio should equal 1. From many repeated control runs we find $A/R = 1 \pm 0.2$. The data in Figure 4 illustrate one set of trials representing the average of four individual tracks on two separate plates. The number of animals in the corresponding section in each track was summed and divided by 4. The $(A/R)_i$ was calculated using the average *N* for each section. We have used this as a standard procedure throughout. That is, all the plots in Figures 3-5 are an average of three to four individual tracks and the reported *A/R*s are calculated from the average *N* values. As an

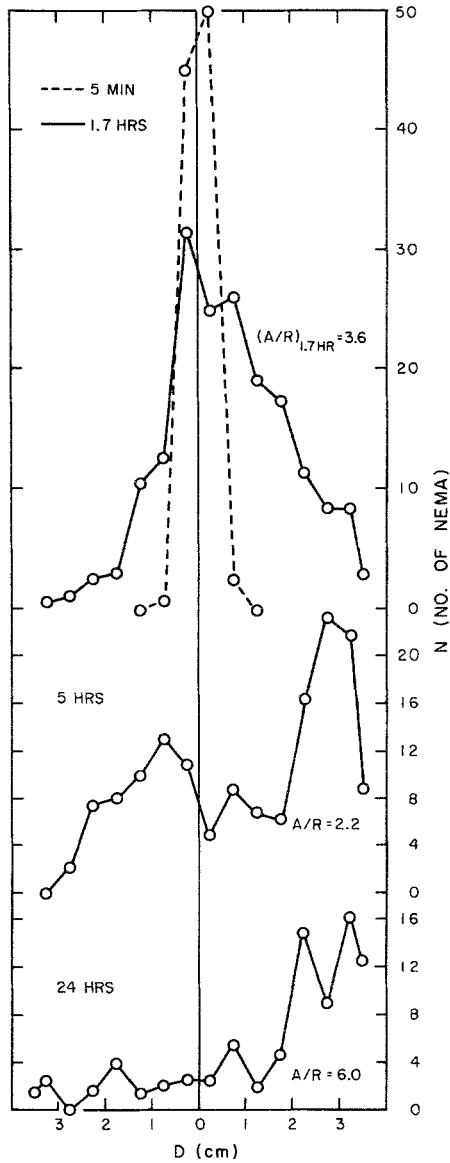


FIG. 3. Response of second-stage larvae of *Meloidogyne incognita* (MiL₂) to cucumber distillate (placed at right side).

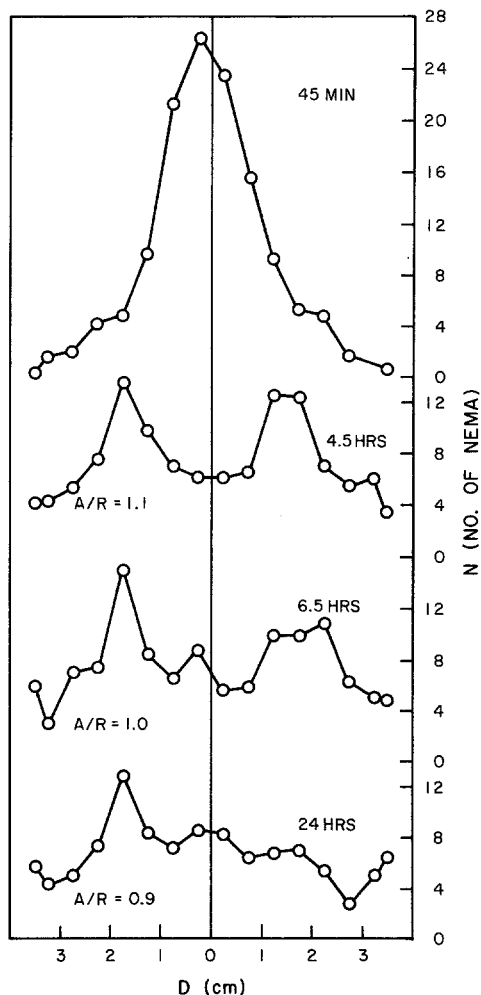


FIG. 4. Control response of MiL_2 .

illustration of reproducibility, the A/R values for each track at 6.5 hr in Figure 4 are 1.0, 1.1, 0.8, and 1.2 (average, 1.0 ± 0.2). For controls, the averaged A/R values are reproducible within $\pm 20\%$ as indicated above. A repellent fraction with an $(A/R)_5 = 0.33_3$, when tested one month later showed $(A/R)_5 = 0.33_3$. However, we find generally that the precision with the attractant and repellent fraction is also $\pm 20\%$. While statistically valid interpretation may be made with smaller differences, we have chosen to designate an attractant frac-

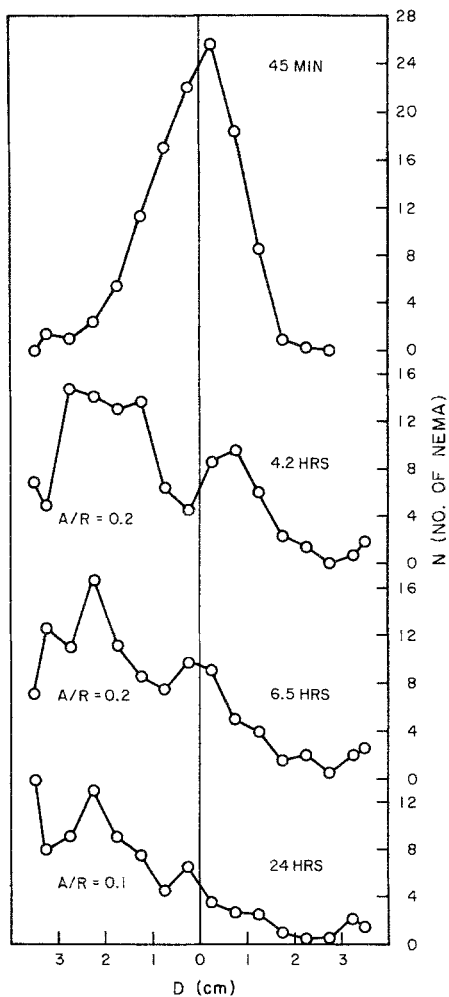


FIG. 5. Response of MiL_2 to cucumber concentrate (placed at right side).

tion as one demonstrating an $(A/R)_5 > 2$ and a repellent fraction as one possessing an $(A/R)_5 < 0.5$. With this nematode the A/R at 5 hr or 20-24 hr could be used. The former is quicker to obtain, and we employ a single count (in replicate) at this time as a standard. These ratios will vary, of course, with the concentration of substance originally employed and the nature of its gradient.

As a precautionary procedure, once the plates are inoculated with nematodes, they are stored in styrofoam boxes and placed in an incubator at 24°C

between counts. This is about 1°C above the average laboratory temperature. This procedure minimizes any thermal response by these animals.

RESULTS AND DISCUSSION

The time when nematodes are inoculated into the tracks can be crucial. Thus, if a suitable gradient has not been established, the nematode sensory apparatus could be either swamped or have nothing to detect. In either case, animals will move away or toward the substance with equal intensity. The ideal situation would be to have a very low concentration of chemical at the center of the track with increasing concentration to one side and none or little to the other. Measured gradients with glycerine, glycine, and *n*-propanol are shown in Figure 2. The correct gradient pattern is established in 22–24 hr for these water-soluble substances. Thus, we have arbitrarily chosen 22 ± 2 hr as the time interval (ΔT) between addition of test fraction and inoculation of the nematodes. For unknown volatiles we have varied ΔT from 5 to 24 hr. Best results were obtained with ΔT s of 20–24 hr. With *n*-propanol a gradient was established even though 80% of the original material was lost to the atmosphere in this time.

Typical results with two fractions from cucumber roots and a control set are shown in Figures 3–5. Appropriate *A/R*s are in the figures; each plot represents an average count from three to four individual tracks.

There are several notable features. First of all, the control runs exhibit an *A/R* at all times of approximately one. For this set, $A/R = 1 \pm 0.1$ but from many experiments of this kind, we find the general range to be 1 ± 0.2 . Figures 3–5 also illustrate the development of population densities with time. As noted in the experimental section, a replicated count at 4.5–5 hr reliably assesses the nematode response. While counts can be taken at any time, we have chosen 5 hr as standard for the routine assessment of characteristic response. Different larval stages and different species may react at different rates. An initial qualitative scan with time is essential for fixing a routine regimen. Responses can also vary with the viability of a population. For example, in some runs, the animals can be dead at the 24 hr count or sooner. The best time is the shortest that allows the response to be reliably assessed. With this nematode, repeat counts at 10 min, 1, 2, 4, 6, 12, and 24 hr established the 4- to 6-hr period as the best counting time.

Figure 3 illustrates the attraction of *Mi* larvae to a fraction of the “distillate” from cucumber roots. Clearly, the 1-hr count was already indicative of the response. The *A/R* ratio does increase at longer times, but it is not necessary to wait 24 hr for the pattern to emerge.

Figure 5 depicts the response of the second-stage larvae to a repellent frac-

tion obtained from the "concentrate." Again, a clear response is discernible in 4–6 hr.

Finally it should be noted that neither of these cucumber fractions increases the speed of nematode movement, that is, the population densities at 4.5 hr in these illustrations are greatest at 1.5 cm in all cases. With repeated measurements we find the average rate of locomotion of this larval stage is ~ 1.5 cm/5 hr. or 0.3 cm/hr. This result is in agreement with those assessed by more elaborate means (Pline and Dusenbery, 1987). On the other hand, if a given substance were to cause an increase in the rate of movement, the assay would have the capacity to detect it.

It should be emphasized that pheromones (Bone, 1980) could play a role in the movements observed but they cannot explain them; that is, at the outset and in the early stages, even numbers of animals are left and right of center. Moreover, while the responses of different populations may differ, these factors too are eliminated from significance by the experimental design. Thus a slower or longer time may be required for the plate to "develop," but any observed response is valid. In all cases, in every track, a population is essentially run against itself. In addition, "controls" can be run on the same plate.

In summary, this bioassay offers a clear interpretation of attraction or repulsion that can be quantified. Thus, with known structures, precise gradients and relative attraction indices can be established with specific animals. Moreover, the method can be applied to the detection of unknown attractants and repellents. We are currently exploring the nature of these unknown substances, and we are beginning to build a base of responses of second-stage larvae of *Meloidogyne incognita* and other nematodes and larval stages to a variety of known substances.

Surprisingly, different fractions of the same plant root contain both an attractant and a repellent. The former is volatile (steam distillable). The latter is not. Speculation as to the significance of this in the life cycle of the plant-nematode interaction is appealing but an explanation awaits the structure of the substances and the timing of their generation.

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STRIKE-INDUCED CHEMOSENSORY SEARCHING OCCURS IN LIZARDS

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Abstract—Strike-induced chemosensory searching (SICS), previously known only in snakes, is experimentally demonstrated in a lizard, *Varanus exanthematicus*. Tongue-flicking rate was significantly greater after striking the prey than following three control conditions. The occurrence of SICS in a varanid lizard suggests that SICS may serve to help relocate dropped or escaped prey not only in snakes, but in other squamates that use the tongue as a chemosensory sampling device during foraging. This in turn suggests the need for further studies of the taxonomic distribution of SICS in squamates and of its relationship to tongue use during foraging and feeding.

Key Words—Lacertilia, *Varanus exanthematicus*, Varanidae, prey odor, tongue-flicking, chemosensory searching.

INTRODUCTION

Strike-induced chemosensory searching (SICS), an elevated tongue-flicking rate observed in snakes following biting, presumably helps these predators to find and follow odor trails of released or escaped prey. It is distinct from trailing itself, being restricted to the increased tongue-flicking rate (Chiszar et al., 1983). The term SICS may thus be interpreted as a poststrike elevation in tongue-flicking rate with no necessary implication that SICS facilitates location of prey by lizards. The elevation in tongue-flicking rate is a specific sequel of striking; rattlesnakes show much higher tongue-flick rates after striking prey than after detecting prey odors (Chiszar and Scudder, 1980). Rattlesnakes also follow scent trails of prey with higher probability after striking and envenomating than after merely seeing (and perhaps smelling by primary olfaction) the prey with-

out striking (Golan et al., 1982). SICS has been studied primarily in venomous snakes that employ a strike-release-trail strategy, which allows them to eat dangerous prey with a greatly reduced chance of being injured (e.g., Chiszar and Scudder, 1980; Chiszar et al., 1982, 1983, 1985, 1986; O'Connell et al., 1982, 1985; Radcliffe et al., 1986).

SICS has also been demonstrated recently in two species of nonvenomous snakes that do not use a strike-release-trail strategy (Cooper et al., 1988). Thus, SICS may have evolved initially to facilitate location of prey known to be nearby, prey that escaped after being bitten. If this hypothesis is correct, SICS might well occur in any squamate that uses the lingual-vomer nasal system extensively during foraging. Occurrence of SICS in a widely foraging lizard such as a monitor (*Varanus*) would be consistent with this hypothesis but would greatly diminish the tenability of the hypothesis that SICS evolved initially in venomous snakes as part of the strike-release-trail strategy.

It was felt that varanids were the lizards most likely to exhibit SICS because varanids have advanced snakelike tongue structure and function, tongue-flick extensively during social situations and during active foraging, and accurately discriminate prey odors from control odors in tongue-flicking tests. Members of the family Varanidae are noted for tongue-flicking during social encounters, especially at their outsets (Auffenberg, 1981; Moehn, 1984; Davis et al., 1986), and during active foraging (Vogel, 1979; Auffenberg, 1981, 1984). The mechanism of transport of chemical cues from the tongue to the vomeronasal organ has not been directly demonstrated in any lizard and the tongue's tips appear not to enter the vomeronasal cavity in monitors (Oelofsen and van den Heever, 1979). However, molecular transfer from tongue to vomeronasal epithelium has been shown in the garter snake (*Thamnophis sirtalis*) (Halpern and Kubie, 1980). Furthermore, there is ample evidence that lizards of several families respond to prey odors and conspecific odors detected by tongue-flicking (e.g., Burghardt, 1973; Duvall, 1979; Simon, 1983; Cooper and Vitt, 1984, 1986; Von Achen and Rakestraw, 1984). In the only laboratory study of varanid responses to prey odors, *V. exanthematicus* fed a consistent diet of mice (*Mus musculus*) in the laboratory show significantly higher tongue-flick attack scores in response to cotton-tipped applicators bearing mouse odors than to those bearing water or cologne (Cooper, 1988).

Varanid tongues appear to be more structurally and functionally specialized as chemosensory sampling devices for the vomeronasal system than those of other lizards. The lingual structure is more similar to that of snakes than that of other lizards in being long and narrow with highly developed tines (McDowell, 1972) and in being devoid of taste buds (Schwenk, 1985). In *V. exanthematicus* the tongue is primarily a chemosensory sampling device that plays no part in food transport during feeding (Smith, 1982). During tongue-flicking, a greater relative area of air is sampled by varanids than by other

lizards. The relative area sampled is comparable to or greater than that of several species of advanced snakes (Gove, 1979).

This study was designed to determine whether SICS occurs in the savannah monitor, *Varanus exanthematicus*. Because this lizard does not normally release living prey after biting, it was necessary to pull the prey from the lizard's mouth. This further required an experimental control for effects on tongue-flicking rate of handling the lizard in addition to the usual controls for disturbance caused by opening the cage, presence of the experimenter, and visual prey cues.

METHODS AND MATERIALS

Seven savannah monitors obtained from a commercial dealer were between 284 and 352 mm snout-vent length. Although these lizards appear to feed primarily on invertebrates in the field (Cisse, 1972), they readily consume mice (*Mus musculus*) in the laboratory. The lizards were housed in a laboratory building having translucent walls and a retractable roof that was opened on clear days. They were housed individually in $49 \times 49 \times 32$ cm glass terraria. Each terrarium contained a sand substrate and water bowl and was covered by a hardware cloth top. Prior to the experiment, the lizards were fed almost exclusively living adult mice.

Each individual was tested in four stimulus conditions, one per day, in random sequence, on May 25-29, 1987, at temperatures from 32.8 to 34.5°C; no tests were conducted on May 27. One condition was a disturbance control (forceps condition) for effects of the presence of the experimenter's hand and opening the cage on tongue-flicking rate. In this condition, the cage top was opened and the experimenter's hand and feeding forceps were placed in the cage for 10 sec and then removed. The ends of the forceps were positioned approximately 10 cm anterior to the monitor's head in this and the other conditions. The second experimental condition consisted of visual presentation of a living adult mouse (mouse condition) for 10 sec without allowing the lizard to closely approach or attack. If the lizard attempted to attack, the mouse was immediately withdrawn. The third stimulus condition served as a control for the effects of pulling bitten prey and lizard apart (pull condition). In this condition, the lizard was pulled away from the mouse just before biting the mouse. When immediate attack seemed probable, as indicated by the lizard's approach and posturing, the experimenter rapidly withdrew the mouse and simultaneously grasped the lizard, pulling it away from the mouse to prevent any oral contact. In the fourth stimulus condition, the mouse was pulled out of the lizard's mouth immediately after having been bitten (strike condition).

Tongue-flicks were counted in all conditions for 2 min after termination of the experimental stimulus. Data were analyzed for homogeneity of variance by

Hartley's test, were square-root transformed, and then were analyzed by a repeated measures design (randomized blocks). Individual comparisons among means were made using Newman-Keuls tests (Winer, 1962). Statistical tests were two-tailed with $\alpha = 0.05$.

Although counterbalancing the order of conditions would have been preferable to randomization, too few specimens were available. The sequences actually used showed little deviation from an incompletely counterbalanced design. In the first two and second two trials, the conditions occurred with the following frequencies: forceps condition (3, 3), mouse condition (2, 4), pull condition (3, 3), and strike condition (2, 4). Sequence had no effect on rank tongue-flick rate for individuals. The two lizards in which the strike condition came last had much higher rates in the strike trial than in the others, indicating that the effect does not disappear due to rapid habituation to the general experimental situation.

Testing for the presence of SICS is a difficult experimental problem because handling or similar mechanical disturbance cannot be readily avoided. The experimental design used here seems drastic at first encounter. It is initially somewhat disturbing or offensive because experimenters usually take great pains to avoid disturbing subjects in behavioral studies. Two problems are that (1) the increase in tongue-flicking rate attributed to striking could have merely represented greater disturbance in the strike condition than in the other conditions and (2) there is no adequate control for tactile stimulation to the mouth resulting from pulling the prey out of it. In the study on SICS in nonvenomous snakes, these problems were addressed by determining tongue-flick rates in garter snakes in a control study. In each trial, the experimenter picked up a snake and then forced into its mouth a cotton-tipped applicator bearing either distilled water only or distilled water plus prey odor. Tactile stimuli and mechanical disturbance were identical in the two conditions, yet the snakes emitted much higher tongue-flick rates in the 5 min following the fish odor presentation (Cooper et al., 1988). Thus, it appears that neither mechanical disturbance nor tactile stimulation to the mouth produce spurious indication of SICS and that the experimental design used is appropriate.

Pulling the monitors by hand is an essential control condition for this experiment because separating the lizards from bitten prey required pulling the prey out of their mouths by simultaneously grasping lizard and prey. Pulling is a rather drastic disturbance in both the strike and pull conditions. Grasping a snake in itself may produce an increase in tongue-flicking rate (Scudder and Burghardt, 1983). In a study of SICS in garter snakes (*Thamnophis sirtalis parietalis*) and corn snakes (*Elaphe g. guttata*), pulling the snakes and visually presenting prey induced roughly equal increases in tongue-flick rate (Cooper et al., 1988). Importantly, in both species, a significant further increase occurred when the snakes were allowed to strike prior to being pulled.

RESULTS

The monitors tongue-flicked in all conditions, often at substantial rates when exposed to prey. There was substantial behavioral variation among individuals in tongue-flicking rates, with one individual having the highest rate in three of the four conditions and another the lowest in two of four. Attack behavior also varied in its dependence on chemical cues. One lizard attacked immediately in all conditions, with no apparent chemosensory investigation. Its data were discarded, reducing the sample size for analysis to six. Tongue-flicking rates in the 2 mins following stimulus removal differed greatly among treatments, with the highest rates being elicited by strikes and the lowest by merely opening the cage and presenting a hand (Figure 1).

Because between-condition variances were not homogeneous (Hartley's $F_{\max} = 34.17$; $df = 4, 5$; $P < 0.05$), data were subjected to square-root transformation. Variances of the transformed data were homogeneous ($F_{\max} = 9.25$; $df = 4, 5$; $P > 0.05$). Using transformed data, the main treatment effect was

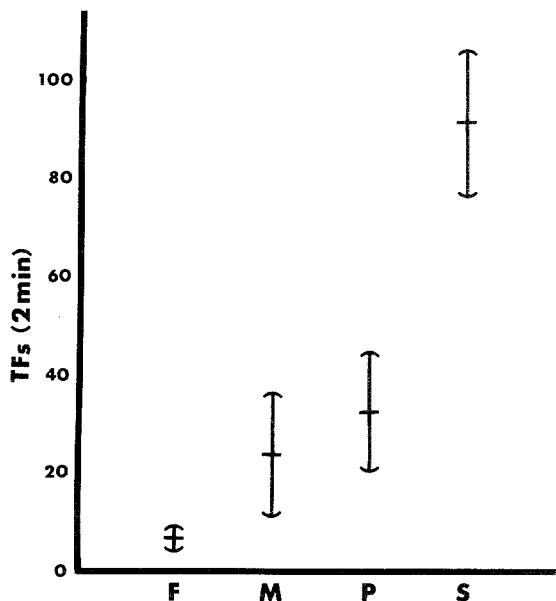


FIG. 1. Tongue-flicks emitted by *Varanus exanthematicus* in the 2 min following removal of the experimental stimuli. F = presentation of empty forceps for 10 sec. M = presentation of forceps and mouse for 10 sec. P = pulling lizard away from prey just prior to strike. S = lizard allowed to strike prey, but prey then removed. Data shown are means (horizontal lines) ± 1 SE.

highly significant ($F = 20.54$; $df = 3, 15$; $P < 0.001$). Newman-Keuls tests revealed that the mean tongue-flicking rate following striking was significantly greater than that in each of the other conditions at $P < 0.01$. Visual presentation of the mouse plus pulling the lizard away from mouse when striking was imminent (pull condition) induced significantly higher tongue-flicking rates than did the disturbance control (forceps condition) without prey ($P < 0.05$), but the substantial difference between rates for visual presentation alone (mouse condition) and for disturbance (forceps condition) did not quite attain significance. Neither did the rates for visual presentation of prey (mouse condition) and visual presentation plus pulling the lizard (pull condition) differ significantly.

DISCUSSION

The primary finding is that SICS occurs in a lizard. A poststriking increase in tongue-flicking rate greater than that attributable to disturbance associated with entering the cage, visual and possibly airborne prey odor cues, or handling of the lizard is evident from the individual comparisons between treatment means. SICS has been known until very recently only in venomous snakes, in which it occurs in numerous viperid and elapid species. The first hints that it might also occur in nonvenomous snakes were that garter snakes showed higher tongue-flicking rate to swabs following trials in which they struck than after trials not involving striking (Burghardt, 1969) and bull snakes (*Pituophis melanoleucus*) showed elevated tongue-flicking rates after swallowing (Chiszar et al., 1980). SICS has recently been discovered in at least two nonvenomous colubrid snakes (Cooper et al., 1988), a colubrine constrictor (*Elaphe guttata*), and a natricine nonconstrictor (*Thamnophis sirtalis*). That SICS has evolved de novo in varanid lizards and three ophidian lines seems highly unlikely. Thus, SICS may be primitive in these groups rather than having evolved convergently to support a strike-release-trail strategy in viperids and certain elapids.

If, as appears to be the case, SICS did not evolve initially to support a strike-release-trail strategy, other functions must be sought in varanid lizards and nonvenomous snakes. A lizard or snake, venomous or nonvenomous, biting a prey item may receive oral chemical stimuli sufficient to locate and identify the prey after it has escaped or been dropped. By using the tongue to gather chemical stimuli from its environment for vomeronasal analysis, a squamate could increase its chances of relocating a prey item. It is because the prey is known to be nearby and perhaps injured that the intensive chemical monitoring of SICS is adaptive. In this view, SICS was probably present in snakes prior to adoption of the strike-release-trail strategy, but was immediately useful in locating and following the scent trail of prey released after envenomation (Cooper et al., 1988). Although SICS is probably homologous in lizards, non-

venomous snakes, and venomous snakes, its features may have been evolutionarily molded to enhance its function in the strike-release-trail context. Quantitative comparisons of SICS parameters in venomous and nonvenomous snakes should reveal any such changes. It should be noted, however, that further experiments are needed to establish that the increased tongue-flicking rate actually functions to help locate prey.

Given that SICS is shared by snakes and varanid lizards, its taxonomic distribution among lizard families is of interest. SICS may occur in numerous families, but if its role is primarily location of escaped prey, as hypothesized, it is likely to be fully expressed primarily in groups of active foragers that use the tongue to locate prey. It may well be entirely absent in lizard families such as Iguanidae (e.g., Evans, 1961; Simon et al., 1981, for *Sceloporus jarrovi*) and Agamidae (Cooper, unpublished data), which do not appear to use the tongue to find prey. According to one hypothesis, snakes originated from platanotan stock (McDowell, 1972), i.e., the lizard families Helodermatidae, Varanidae, Lanthnotidae, and three extinct related families. This hypothesis would be supported if SICS among lizards were restricted to this lineage.

Responses of lizards and nonvenomous snakes to the experimental conditions used for varanids are congruent. In both species of nonvenomous colubrids (Cooper et al., 1988) and in *V. exanthematicus*, visual presentation of prey and pulling the predator away from the prey induced higher tongue-flicking rates than did the disturbance control; striking induced a still greater rate. The only difference of significance in the pattern is that the tongue-flicking rate elicited by visual presentation was not quite significantly greater than that following disturbance in the lizard but was so in the snakes. The similarity of response by lizards and snakes in these conditions further hints at possible homology of SICS in varanid lizards and snakes. It should be recognized that proprioceptive cues and oral tactile stimulation produced by striking and thus consistently associated with SICS are absent in the control conditions. Thus, the stimuli eliciting SICS are not necessarily chemical but could be at least in part tactile.

The tongue-flicking rate during the 2 min following striking in *V. exanthematicus* (46/min) was within the range reported for venomous snakes, but below that recorded for two nonvenomous colubrids. Tongue-flicking rates during the same interval range vary from slightly under 20 to over 70/min for various viperids (Chiszar et al., 1980, 1983, 1985; O'Connell et al., 1981, 1982), between 40 and 60/min for several elapids (O'Connell et al., 1985, Radcliffe et al., 1986), and for the nonvenomous colubrids *Elaphe g. guttata* and *Thamnophis s. sirtalis*, ca. 60 and 72.5/min (Cooper et al., 1988). The large rate differences within families and overlaps among families suggest that varanid lizards fall within the normal response range for snakes. However, further interpretation is difficult because numerous features other than inherited taxonomic differences presumably affect these rates, including temperature,

hunger, prey type, prey odor, prior diet, and experimental treatments, among others.

Visual prey cues plus possible airborne prey odors elicited tongue-flick rates considerably higher than did disturbance without such cues, but the difference was not quite statistically distinct (Newman-Keuls observed value = 9.92; value for significance at 0.05 = 11.65). Because the sample size was small and the variability in tongue-flicking rate high, it seems likely that the large difference in tongue-flicking rates represents a true inequality of response. In all three families of snakes studied, brief visual presentation of prey induces elevated tongue-flicking rates (e.g., Chiszar et al., 1985; O'Connell et al., 1985; Radcliffe et al., 1986; Cooper et al., 1988). Activation of tongue-flicking by visual prey stimuli is presumably an investigatory behavior functioning to confirm the visual cues and, if the prey is no longer in sight, to increase the probability of relocating it.

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FINE-SCALE VARIABILITY OF LANOSOL AND ITS
DISULFATE ESTER IN THE TEMPERATE RED
ALGA *Neorhodomela larix*

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Abstract—Aqueous extraction and HPLC separation techniques were used to quantify two major bromophenols naturally present in the red alga *Neorhodomela larix*: lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) and its 1,4-disulfate ester. Maximum concentrations of each compound were as high as 1.5% on a wet mass basis. The within-plant distributions of lanosol and its ester were highly variable on centimeter scales: adjacent portions often varied by an order of magnitude in bromophenol content. Some bromophenol variation was related to algal phase, location within the algal thallus, and reproductive status. Bromophenol concentrations were higher in exterior vegetative regions and some reproductive structures (cystocarps and tetrasporangial branchlets) than in interior vegetative regions or male reproductive structures (spermatangial stichidia). In contrast to results reported for harvested *N. larix*, there was no evidence that the intact *in situ* algae released either compound into seawater.

Key Words—*Neorhodomela larix*, red alga, lanosol, 2,3-dibromo-4,5-dihydroxybenzyl alcohol, lanosol 1,4-disulfate ester.

INTRODUCTION

The abundance of bromophenols within the red alga *Neorhodomela (Rhodomela) larix* (Turner) Masuda (Masuda, 1982) implies important roles for the compounds, perhaps as deterrents to grazing or fouling. One barrier to under-

standing the ecological effects of the bromophenols has been uncertainty about precisely which and how many bromophenols are present within the intact algae. One bromophenol extractable from *N. larix* is lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol). Other variants of lanosol described from *N. larix*, especially organic ethers, appear to have been artifacts of extraction in organic solvents (Scheuer, 1973; Glombitza and Stoffelen, 1972; Weinstein et al., 1975; Saenger et al., 1976; Higa, 1981). In particular, 2,3-dibromo-4,5-dihydroxybenzaldehyde, which was abundant in ethyl acetate extracts (Phillips and Towers, 1981), was not identified in subsequent methanol extracts (Phillips and Towers, 1982a). Weinstein et al. (1975) argued that lanosol itself was a product of hydrolysis during extraction and that the only bromophenol actually present in *N. larix* was the disulfate ester, 2,3-dibromo-5-hydroxybenzyl-1',4'-disulfate, presumably as the potassium salt. We have developed aqueous extraction and separation techniques that reveal the simultaneous presence of lanosol and the disulfate ester in *N. larix*. We have also observed millimeter- to centimeter-scale variability in the concentrations of both compounds within the algal thalli. Contrary to previous work with harvested algae (Phillips and Towers, 1982b), we find no evidence that either compound is released into solution by intact plants *in situ*.

METHODS AND MATERIALS

Specimens of *N. larix* were collected in 1986 and 1987 from Boiler Bay, 17 miles north of Newport on the central coast of Oregon. Algae were carried in plastic bags on ice to Oregon State University's Hatfield Marine Science Center at Newport, where they were examined under a dissecting scope to determine life-history phase (male gametophyte, cystocarpic female gametophyte, tetrasporophyte) and reproductive status (relative abundance and condition of reproductive structures). Thalli were cut transversely into roughly centimeter-long sections. Each section was measured, blotted dry, weighed, and then frozen individually. If macroscopic epiphytes were present on a section, their abundance was recorded and they were removed before the section was weighed. Of the approximately 30 sections produced from each plant, we analyzed a subset that included all main axis and branch tips, all sections of at least one branch, at least one section from base and mid-axis regions, one section from each of the reproductive status categories, and all sections whose reproductive status was observed to be intermediate or higher. In June 1987, additional algal sections were further dissected into (1) the cortex (outer pigmented region) of the main axis, (2) the medulla (inner, nonpigmented region) of the main axis, (3) the spikelike ultimate branchlets of the main axis and larger lateral branches, and (4) reproductive structures. Reproductive structures

included spermatangial stichidia from males, cystocarps from females, and stichidial-like branchlets bearing tetraspores from tetrasporophytes.

The frozen sections, weighing 10–150 mg, were homogenized for 4 min in 150 μ l of distilled deionized water and then for 2 min more following the addition of 1 ml of phosphate–borate buffer (KH_2PO_4 , H_3BO_4 , and KCl each at 50 mM, adjusted to pH 6.9 with HCl). Homogenization was done by hand in 5-ml glass tissue grinders. The pH of the homogenate was adjusted to 10.0 with NaOH to keep the phenols in solution as the homogenate was centrifuged and then filtered (through 0.2 μ m PTFE membranes). The filtrate was then reacidified to pH 6.0 with 2 N HCl, a pH at which the phenols were stable for at least 24 hr. An internal standard, 2,3-dichlorophenol, was added to all samples before homogenization to monitor recovery of halogenated phenols by this extraction technique. Residual algal material from an aqueous extraction was homogenized again in chloroform to test aqueous extraction efficiency. The chloroform extracts were then analyzed using the same chromatographic conditions as used for the aqueous extracts.

Subsamples (200 μ l) of the acidified aqueous solutions were chromatographed on a Hamilton PRP-1 column (7.0 mm diameter, 30.5 cm long, with 10 μ m particle size). The bromophenols were separated using combined gradients of pH (acidic to basic) and solvent (aqueous to organic); mobile-phase compositions, gradient parameters, and flow rates are given in Table 1. Once separated, the bromophenols were detected by their absorbance at 280 nm, near

TABLE 1. ELUTION GRADIENTS AND MOBILE-PHASE COMPOSITIONS FOR CHROMATOGRAPHIC SEPARATIONS OF BROMOPHENOLS EXTRACTED FROM *N. larix*

Gradient program		Mobile phase ^a			
Step	Time (min)	%A	%B	%C	%D
0	2.0	80			20
1	2.0	80			20
2	2.0		80		20
3	4.0		40		60
4	5.0		40		60
5	2.0			100	
6	4.0			100	
7	2.0	80			20

^aCompositions of the four mobile phase solutions were: A, 10 mM NaH_2PO_4 with 18 mM ammonium acetate, pH 4.5; B, 10 mM Na_2HPO_4 with 18 mM ammonium acetate, pH 10.0; C, 90% methanol, 10% distilled deionized water; D, 100% acetonitrile. All changes in composition were by linear gradients.

λ_{\max} for many bromophenols. The basic mobile phases (final pH ≥ 10.0) enhanced detection: extinction coefficients of many phenols increase greatly in basic solutions. Disulfate ester was identified by coretention with an authentic standard (graciously provided by J. Craigie) (Figure 1) and by comparison of its UV spectrum to authentic material; it was quantified using response factors generated from the authentic material. Lanosol was identified and quantified in the same manner (Figure 1) using standards derived from the ester standard by 15-minute hydrolysis at pH 1.2 and 100°C. Disulfate ester and lanosol were

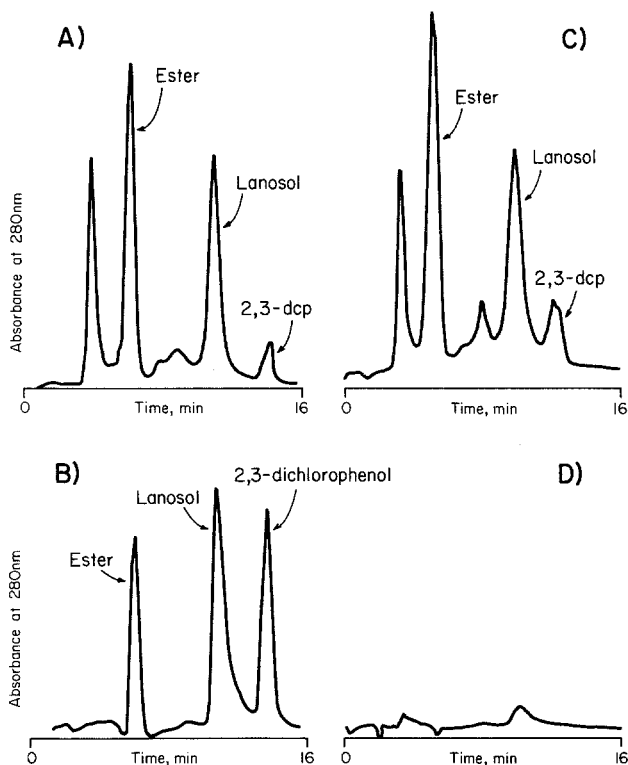


FIG. 1. Chromatograms of bromophenols extracted from *N. larix*. (A) Aqueous extract of a cystocarpic female collected in July 1986 showing ester, lanosol, and 2,3-dichlorophenol peaks as well as peak of more polar material eluting before the ester. (B) Standards: 15 μg ester, 29.8 μg lanosol, and 3.7 μg 2,3-dichlorophenol per injection. (C) Aqueous extract of a reproductive tetrasporophyte collected in October 1986. Ester and lanosol concentrations per 200 μl injection were 22.8 and 20.7 μg , respectively, from a 35.8-mg section of the alga, giving concentrations of 3504 and 3177 $\mu\text{g/g}$ fresh weight of ester and lanosol, respectively. (D) Chloroform extract of the residual algal material from C. Neither ester nor lanosol was evident above the gradient baseline values.

added to some algal samples before homogenization and then processed with and without acidic hydrolysis to determine the stability of the two compounds under the extraction conditions. Bromophenol concentration data were subjected to two multivariate analyses of variance, with phase (male, female, tetrasporophyte) and thallus part (tips, midsections, basal portions) or phase and reproductive status as variables, respectively.

We considered several options for storing algae between collection and analysis. In one comparison, extracts of frozen individual algal pieces had more lanosol and ester than extracts of refrigerated pieces, suggesting that freezing improved extraction, presumably by disrupting plant structure. Frozen algae were easier to homogenize than fresh or refrigerated plants. In a second comparison, algae frozen in a mass (20–50 g wet mass), rather than as individual pieces, showed variable and occasionally increased lanosol and ester concentrations with time, perhaps because biological activity was not quickly or thoroughly arrested in the time required to completely freeze the larger mass of algae. Based on these comparisons, we chose to freeze algal pieces individually until analysis.

Immersion experiments were conducted in the field in April, July, and August of 1987 to determine whether the algae released bromophenols after exposure to air at low tide. We gently immersed single axes or branches of exposed intact attached *N. larix* for intervals of 1–120 min in tubes containing 10 ml of ambient seawater. After the algae were removed from the tubes, the tubes and their liquid contents were kept cool and dark during transport to the laboratory. There, we added 3.3 ml of saturated KCl in seawater to the 10-ml immersion solutions to decrease the solubility of the bromophenols. The entire solutions were then passed through phenyl solid-phase extraction columns (3 ml column volume, 200 mg sorbent). The columns were rinsed with 400 μ l hexane to remove some of the water and associated salts. Phenols retained on the extraction columns were eluted with three 333- μ l volumes of methanol and chromatographed as above, after centrifugation to remove precipitated salts remaining in the methanol. Disulfate ester was added to some solutions as the algae were immersed to determine extraction efficiencies and stability during collection and transport. In August, several plants were scraped and nicked slightly with razor blades to determine whether abrasions or incisions might induce bromophenol release.

RESULTS

The combination of aqueous extraction with high pH chromatographic mobile phases resulted in a sensitive and effective technique for the identification and quantification of bromophenols. Detection limits under these conditions were ≤ 10 ng for both lanosol and the disulfate ester. This sensitivity

allowed bromophenol measurements of algal pieces as small as 5–10 mg wet (damp dry) mass. The extraction itself was efficient: homogenization times longer than the 6 min reported here did not result in greater recovery of either lanosol or its disulfate ester. Grinding residual algal materials in chloroform after aqueous homogenization extracted no additional lanosol, ester, or other UV-absorbing materials (Figure 1D). Individual sections of frozen algae subsampled over the course of three months showed no significant changes in lanosol or ester content (see Table 3, discussed below).

Recoveries of the bromophenols from the extraction process were reliably high. Recoveries of the internal standard, 2,3-dichlorophenol, added as part of the homogenization buffer, were consistently 61–68% (Table 2). The missing 2,3-dichlorophenol was probably adsorbed to particulate fractions or to filter or container surfaces. Such losses were not evident when lanosol or the disulfate ester were added directly to algal samples (Table 3, below); both brominated compounds are more hydrophilic than 2,3-dichlorophenol.

Lanosol and the disulfate ester were found in every section of all seven plants measured from the April collections (Table 2) and in all dissected portions from the June collections. The presence of lanosol was not an artifact of

TABLE 2. ESTER AND LANOSOL CONCENTRATIONS IN SECTIONS OF SEVEN *N. larix* PLANTS COLLECTED APRIL 1987

	Males		Females			Tetrasporphytes	
	Plant 1	Plant 2	Plant 1	Plant 2	Plant 3	Plant 1	Plant 2
Pieces ^a	19(30)	16(27)	20(39)	20(39)	33(33)	13(33)	10(22)
Ester ($\mu\text{g}/\text{g}^b$)							
Mean	3369	3729	5265	5899	3991	2223	1542
SE	464	407	684	666	393	337	178
Max	8205	8714	9611	16430	9791	4228	2426
Min	416	1753	1014	1987	712	907	896
Lanosol ($\mu\text{g}/\text{g}^b$)							
Mean	4262	5730	6378	5678	5210	5416	5049
SE	317	524	771	589	452	418	395
Max	6356	9840	17510	13977	11222	8943	6965
Min	1395	1429	2741	2579	1068	3128	3504
Dichlorophenol recovery (%) ^c							
Mean	66.9	66.2	60.8	62.8	66.8	68.1	63.6
SE	1.1	1.6	2.1	2.2	1.7	1.9	1.4

^aPieces: number of pieces analyzed, with total number of pieces for the alga in parentheses. Rather than measure all pieces of all plants, pieces were selected for analysis according to criteria given in text.

^bMicrograms per gram wet mass of algae.

^c2,3-Dichlorophenol was an internal standard added before homogenization.

ester hydrolysis during extraction. Disulfate ester added to samples before homogenization was entirely recovered as ester. Lanosol added to samples was likewise entirely recovered as lanosol. When acid hydrolyses were included as part of extraction, the total lanosol resulting from hydrolysis of a subsample of a piece of algae was, with only one exception, very close to the sum of lanosol plus ester (in lanosol equivalents) in another unhydrolyzed subsample (Table 3). These results were remarkable in light of within-thalli variability of the compounds. In addition to demonstrating the simultaneous presence of ester and lanosol in *N. larix* and the complete conversion of the former to the latter by hydrolysis, the results in Table 3 show the temporal stability of the bromophenol concentrations in frozen individual sections of the algae: more than three months elapsed between analyses with and without hydrolysis.

Other UV-absorbing compounds were evident in chromatograms of some of the algal extracts (Figure 1), some with UV spectra characteristic of phenols. An intensely absorbing polar peak was the most frequently observed of these

TABLE 3. TOTAL LANOSOL RESULTING FROM ACIDIC HYDROLYSIS OF SECTIONS OF *N. larix* COMPARED TO SUM OF LANOSOL EQUIVALENTS FROM ESTER PLUS ACTUAL LANOSOL MEASURED LATER WITHOUT HYDROLYSIS IN ANOTHER SIMILAR SECTION OF SAME THALLUS

Thallus ^a	Ester (as lanosol ^b)	Lanosol ($\mu\text{g/g}$ wet mass, actual ^c)	Sum ^d ($\mu\text{g/g}$ wet mass) ^d	Lanosol (hydrolyzed ^e)	Percent ^f	Days ^g
July female	12,186 (6861)	3988	10,849	10,964	99	104
July female	13,092 (7371)	5280	12,651	13,288	95	104
September tetra	3812 (2146)	8000	10,146	10,310	98	106
October tetra	9908 (5578)	4083	9961	9678	100	89
October tetra	13,534 (7620)	3345	10,965	13,566	81	91
October tetra	2135 (1202)	8002	9204	11,107	83	90
October tetra	9077 (5110)	4606	9716	8183	119	90
October tetra	13,019 (7330)	2412	9742	7543	129	92
October tetra	12,052 (6785)	1889	8674	5108	170	91
October tetra	6594 (3712)	3948	7660	6812	112	98

^aAll algae collected in 1986; tetra = tetrasporophyte.

^bValues in parentheses are the amounts of lanosol that would result from complete conversion of the amount of disulfate ester measured, calculated using the ratio of the molecular weights of the ester and lanosol.

^cActual lanosol measured in unhydrolyzed sections.

^dSum = actual lanosol measured plus lanosol equivalents of the ester measured.

^eAmount of total lanosol measured in hydrolyzed plant sections.

^fThe sum of the lanosol and lanosol equivalents measured in unhydrolyzed sections (Sum, column 4) as a percentage of total lanosol measured after hydrolysis in similar sections.

^gNumber of days elapsed between measurements with hydrolysis and measurements without. Algal sections were stored frozen in the interval.

unidentified compounds; others were less absorbant or less abundant and less frequent in occurrence. None of the unidentified compounds had obvious relation to algal part, algal phase, or ester or lanosol concentration.

Amounts of lanosol in single sections of algae collected in April 1987 ranged from 791 to 17509 $\mu\text{g/g}$ wet mass; amounts of disulfate ester ranged from 416 to 16,430 $\mu\text{g/g}$ (Table 2). The maximum total bromophenol (lanosol plus ester) contents of individual sections were 15,000 to more than 30,000 $\mu\text{g/g}$, 1.5–3% by wet mass. If wet mass to dry mass ratios are ≥ 2 (six algal sections we measured had ratios near 5), some sections of these algae contained more than 6% bromophenols by dry weight.

All sections of tetrasporophytes and most sections of male and female gametophytes collected in April 1987 contained more lanosol than ester. The only sections containing much more ester than lanosol were small secondary branches and small developing branches on the main axis of gametophytes (e.g., Figure 2). None of the reproductive sections of tetrasporophytes had especially

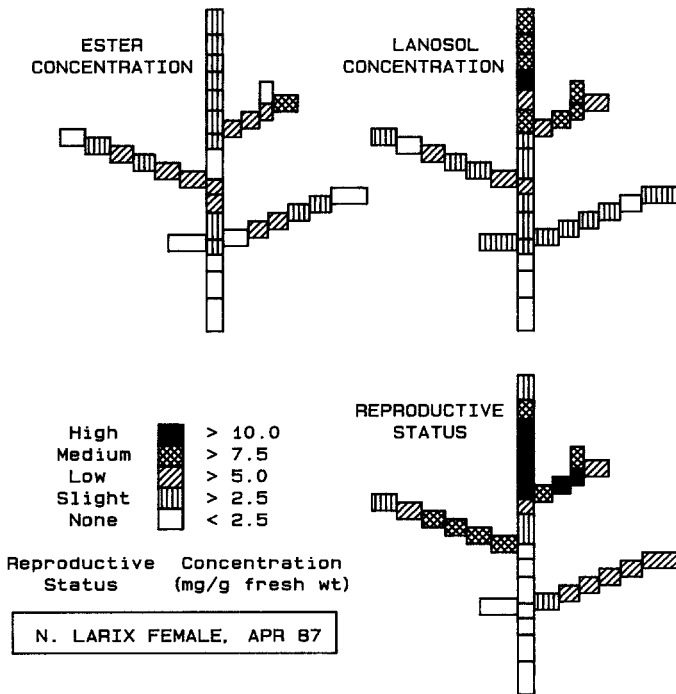


FIG. 2. Computer-generated outline of a cystocarpic female *N. larix* (female 3 in Table 2) collected in April 1987 showing ester and lanosol concentration and reproductive status for each of 33 sections of the alga. Section lengths and branch points in outline are in proportion to actual measurements; the intact alga was 19.2 cm long.

high concentrations of either bromophenol. Some sections of female gametophytes containing cystocarps did have high bromophenol concentrations, especially of lanosol (Figure 2). In some thalli, tips of lateral branches or of the main axis contained high concentrations of the two bromophenols, but in other thalli the highest concentrations were found midway along axes or branches or at the intersection of branches with the main axis. There was, as a consequence, intense fine-scale variability: adjacent 1- to 2-cm-long pieces differed in lanosol or ester content, often by a factor of 2 and sometimes by factors of 10 (Figure 2). The multivariate analyses of variance identified significant differences in ester content according to phase, plant part, and reproductive status, along with significant phase \times part and phase \times reproductive status interaction terms. For lanosol, only the two interaction terms were significant.

Analyses of the dissected portions of *N. larix* collected in June showed that ester and lanosol were largely in the external portions of the vegetative portions of thalli: ultimate branchlets and cortical portions of the main axis had higher concentration ranges of ester and lanosol than did the central medulla of the main axis (Figure 3). Comparison of reproductive structures of these algae indi-

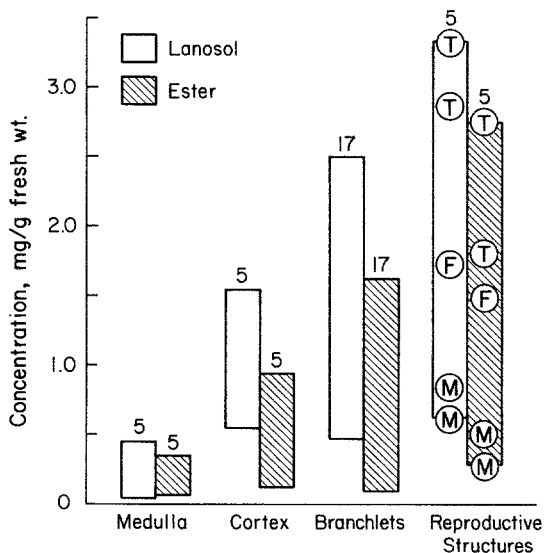


FIG. 3. Lanosol and ester concentrations in reproductive portions of three *N. larix* collected in June 1987: one male, one cystocarpic female, and one reproductive tetrasporophyte. Numbers over the bars indicate the number of samples of each dissected portion analyzed. Symbols indicate the source of the reproductive structures: M = male (spermatangial stichidia), F = female (cystocarps), T = tetrasporophyte (stichidial-like branchlets bearing tetraspores).

TABLE 4. AMOUNTS OF DISULFATE ESTER AND LANOSOL IN SEAWATER IMMERSION SOLUTION, MEASURED IN *In Situ* IMMERSION EXPERIMENTS WITH INTACT *N. larix*

Date	Phase ^a	Immersion time (min) ^b	# Plants (N)	Weight (g) ^c	Ester ($\mu\text{g}/10\text{ ml}$ seawater)	Lanosol ($\mu\text{g}/10\text{ ml}$ seawater)
April 87	T	1-60	6	0.4-1.2	N.D. ^d	N.D.
June 87	T	2-120	5		N.D.	N.D.
August 87	T	60	5		N.D.	N.D.
	T	60	5 ^e	N.D.	5.8 ^f	N.D.

^aT = tetrasporophyte.

^bImmersion times for each individual thallus varied within the range given.

^cWeight of immersed portions of thalli, measured after immersion experiments. Plant portions used in subsequent immersion experiments were of similar size.

^dN.D. = not detectable, with detection limits of 10 ng for ester and lanosol.

^eThese thalli were nicked gently with razor blades to mimic physical damage as might occur from sand, waves, or grazers.

^fValue from the only one of five thalli treated which released detectable amounts of any bromophenol.

cated lowest bromophenol concentrations in male structures and higher concentrations in carposporophytes and tetrasporangial branchlets (Figure 3).

There was no evidence in any of the immersion experiments conducted in April, June, and August of 1987 that lanosol or ester was released by *in situ* intact axes or branches of exposed algae once the axes or branches were immersed in seawater (Table 4). Moreover, neither lanosol or ester was detected in seawater draining naturally across a bed of *N. larix* in April. Ester standards and ester added to the immersion solutions were completely retained by the solid-phase extractions and easily detectable in the subsequent chromatography. Thus, the absence of bromophenols in the immersion solutions seems definitive evidence that the compounds were not released by these intact algae. When thalli in August were intentionally damaged with razor blades to mimic grazing or other physical damage, a slight release of ester was observed in only one of five nicked thalli (Table 4).

DISCUSSION

It is inherently possible that compounds are altered during the extraction and isolation processes involved in analyzing chemical compositions of plants. When the ecological activity of the compounds is under investigation, analyses and subsequent manipulations must be made of the compounds in or close to their native form. It is evident from literature cited in the introduction that sol-

vent extractions have not provided an accurate description of bromophenols intrinsic to, as opposed to extractable from, *N. larix*. The aqueous extraction techniques described here indicated that there were two major bromophenols present simultaneously in these *N. larix*: lanosol and its disulfate ester. Both compounds were stable under these extraction and chromatographic conditions. If hydrolysis was included during extraction, ester was converted to lanosol. Previous investigations employing hydrolysis would not have detected the ester. In addition to lanosol and its ester, other compounds were sporadically present, but those had lesser absorbance and have yet to be identified as bromophenols. It appears, therefore, that the bromophenol products of aqueous extractions of these algae are representative of the intrinsic bromophenol content. Aqueous extractions have the added advantage of being faster and safer than conventional extractions involving organic solvents, although careful control of pH must be maintained throughout.

In addition to improving the accuracy of extraction techniques, we have improved the sensitivity. The improved sensitivity is primarily a result of having the compounds in basic mobile phases as they pass the detector: extinction coefficients of most phenols increase by 10^2 to 10^3 at high pH (Scott, 1964). Using high pH solutions requires polymeric solid phases and increases preventive maintenance of HPLC components in contact with buffers, but the result is much greater within-thallus resolution. The improved resolution is made possible by a large reduction in the amount of algal material needed for measurement, from grams of materials required for previously described organic solvent extractions to milligrams of materials by these aqueous extraction techniques. Not surprisingly, improved resolution reveals chemical patterns and variability previously unrecognized, within and among individual algae according to phase, location, reproductive status, and other factors as yet unidentified. This chemical complexity is on spatial scales that might be recognized by and pertinent to grazers or fouling organisms but that cannot be recognized in analyses of whole thalli.

Neither lanosol nor its ester was uniformly abundant within whole *N. larix* thalli. Furthermore, these compounds did not occur in consistent or predictable gradients in the algae, for example, from base to tip. It appears that there is local production or accumulation of the compounds within the alga, perhaps in response to local growth, reproductive conditions, local grazing, or fouling stimuli. The usual techniques involved in estimating or identifying deterrent effects, such as grinding whole plants for artificial diets or presenting large plant portions in choice experiments, may miss biologically relevant information if within-plant variability of potential deterrent compounds on these scales is not considered.

Although various pharmacological activities have been described for lanosol and to a lesser extent the disulfate ester (Mautner et al., 1953, McLachlan

and Craigie, 1966; Glombitza et al., 1974), we currently do not know the ecological effects of either compound on grazers or epiphytes of *N. larix*. The effects do not seem to involve release of the compounds into solution, as these algae did not release bromophenols. If ester or lanosol concentrations within *N. larix* sampled in the immersion experiments were 1000–10,000 $\mu\text{g/g}$ as in other plants analyzed, release by 1 g of plant material immersed in 10 ml of seawater for an hour must have been less than 0.001–0.0001%/hr in order to be below our 10-ng detection limits. The previous evidence for release of bromophenols by *N. larix* was calculated to be 0.3–1.6%/hr and suggested to be at a maximum in summer months. That evidence came from algae harvested from brackish tidepools, cleaned and washed, transported, rewashed, immersed for 48 hr, then washed again before being reimmersed for measurements of exudation (Phillips and Towers, 1982b). Our measurements were made in the spring and summer directly on algae in the intertidal zone, without harvesting and with minimal handling. *N. larix* exist in a very turbulent environment along the open Oregon coast, conditions that may preclude effective algal influence on surrounding solutions by release of chemicals.

Our observations of fine-scale variation in bromophenol content within *N. larix* come largely from plants collected in April 1987. Within those samples, there were indications that bromophenol content might vary according to reproductive status or growth, both of which vary seasonally. Other factors influencing bromophenol content might be related to tidal height or to latitude. However, investigations of large-scale factors without an appreciation of the considerable fine-scale within-thallus variation in bromophenols could result in confusion or misinterpretation. Now that we are aware of fine-scale variability, we can explore the causes and consequences of variation on that scale and then integrate the fine-scale information into investigations of larger-scale patterns.

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TANNIN-BINDING PROTEINS IN SALIVA OF DEER AND THEIR ABSENCE IN SALIVA OF SHEEP AND CATTLE

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Abstract—A method has been developed for detecting tannin-binding proteins in the saliva of herbivores. The method is simple and requires only small quantities of crude saliva. The saliva of deer, a browsing ruminant, has been compared to that of domestic sheep and cow, which are grazing ruminants. The browser, which normally ingests dietary tannin, produces tannin-binding proteins, while the grazers do not produce such proteins. The tannin-binding protein from deer saliva is a small glycoprotein containing large amounts of proline, glycine, and glutamate/glutamine. The protein is not closely related to the proline-rich salivary proteins found in rats and other nonruminant mammals.

Key Words—Tannin, saliva, ruminant, proline-rich protein, electrophoresis, deer, sheep, cattle, grazers, browsers.

INTRODUCTION

Dietary tannins may affect herbivores by reducing protein digestibility (Rhoades and Cates, 1976; Robbins et al., 1987a) or by systemic toxicity (Dollahite et al., 1966; Martin et al., 1987). Successful herbivores have developed mechanisms for overcoming these adverse effects. For example, some insects have modified digestive tracts in which tannin does not complex protein (Martin et al., 1987). In tree locusts, absorbed phenolics are not toxic but are utilized in

synthesis of the cuticle (Bernays and Woodhead, 1982). Rats synthesize salivary proline-rich proteins (PRPs) in response to tannin-containing diets (Mehansho et al., 1983). PRPs apparently protect rats by binding to the tannin and preventing its interaction with other proteins. Salivary tannin-binding proteins could also minimize absorption of tannins and reduce their toxicity.

We are currently seeking to evaluate differences in the abilities of various ruminants along the grazer-to-browser continuum to consume tannin-containing diets. Grazers consume virtually tannin-free diets and have a minimal ability to tolerate soluble phenolics, while browsers normally consume a variety of phenolic-containing plants and are well prepared to tolerate phenolics (Robbins et al., 1987b). The saliva of mule deer (*Odocoileus hemionus hemionus*), a browser, contains two to three times more nitrogen than the saliva of grazers such as cows or domestic sheep (Robbins et al., 1987b). Deer saliva is more proline rich than cow or sheep saliva and has a greater tannin-binding capacity than the saliva of the grazers (Robbins et al., 1987b). These findings suggest that the tolerance of browsers for dietary tannins may be due, in part, to the production of salivary PRPs. We have developed new methodology that facilitates the detection of tannin-binding proteins in saliva samples and have applied those methods to a detailed characterization of the salivary proteins of deer and sheep.

METHODS AND MATERIALS

Electrophoresis. Nondenaturing, discontinuous, pH 8.3 polyacrylamide gels (Davis, 1964) were used for identification of tannin-binding proteins. The gels were poured in a 1-mm slab gel apparatus using Laemmli's (1970) modification of the method, omitting the sodium dodecyl sulfate (SDS). The acrylamide concentration varied linearly from 7.5% at the top of the gel to 12% at the bottom of the gel, and the bisacrylamide was 0.2% throughout the gel. The stacking gel contained 3.0% acrylamide and 0.08% bisacrylamide. The gels were run at a constant voltage of 300 V for 10–12 hr at 4°C with 0.05% bromophenol blue as the tracking dye.

SDS gels were run to establish whether the composition of saliva changed when animals were fed tannin. The gels were prepared as described by Laemmli (1970). The resolving gel contained 12% acrylamide, 0.3% bisacrylamide, and 0.1% SDS. The stacking gel contained 3.0% acrylamide, 0.08% bisacrylamide, and 0.1% SDS. Samples were prepared by mixing the sample with β -mercaptoethanol, glycerol, SDS, and bromophenol blue and heating the mixture in a boiling water bath for 4 min to denature the proteins. The gels were run at a constant voltage of 90 V for 20 hr at room temperature.

After running either type of electrophoresis, the proteins were fixed in the gels with a mixture of 26% ethanol, 14% formaldehyde, and 60% water for 1 hr, followed by 1–12 hr in a mixture of 50% methanol, 12% acetic acid (Steck et al., 1980). The formaldehyde fixation step is essential for retaining small, proline-rich proteins in the gel throughout the staining procedure. The proteins were then stained with a sensitive silver stain (Merril et al., 1981) or with Gelcode silver stain (Pierce Chemical Co., Rockford, Illinois 61105). The SDS was washed out of SDS gels with a mixture of 10% ethanol and 5% glacial acetic acid before staining.

Assay for Tannin-Binding Proteins. A sample of crude saliva (30 μ l) or an aqueous solution of isolated salivary protein (30 μ l of a 4 μ g/ μ l solution) was mixed with a 50% methanol solution of tannin (10 μ l containing 0.5–5 μ g of tannin), and the mixture was incubated for 6–9 hr at 4°C. The sample was centrifuged at 800g and the supernatant mixed with 5 μ l of glycerol and 5 μ l of 0.05% bromophenol blue before loading onto a native gradient gel. Control samples were mixed with 50% methanol but no tannin. In the presence of tannin, tannin-binding proteins did not enter the gel or had altered electrophoretic mobility. These proteins could thus be identified by comparing the electrophoretic pattern of proteins in control samples to the pattern in the presence of tannin.

A variety of tannins were used to establish whether the tannin-binding proteins exhibited tannin-specific binding. Quebracho tannin (Asquith and Butler, 1985), sorghum tannin (Hagerman and Butler, 1980), and tannic acid (Hagerman and Klucher, 1986) were purified by a combination of ethyl acetate extraction and Sephadex LH-20 chromatography. Tannin-containing extracts were prepared using 50% methanol (3.7 ml/g tissue) and a microcolumn extraction technique (Hagerman, 1988). Plants used included maple leaves (*Acer rubrum*), fireweed flowers (*Epilobium angustifolium*), and red-osier dogwood leaves (*Cornus stolonifera*) (Robbins et al., 1987a).

Isolation of Tannin-Binding Protein. Saliva samples were collected from a resting female deer with surgically constructed esophageal fistula and immediately frozen. After thawing, the saliva was mixed with an equal volume of 10% (w/v) trichloroacetic acid. Twenty minutes later the mixture was centrifuged for 15 min at 10,000g and the precipitate was discarded. The cloudy supernatant liquid was brought to pH 7 and clarified by addition of a few drops of a concentrated solution of sodium hydroxide. The supernatant was dialyzed against distilled water at 4°C and lyophilized to produce a white or tan powder consisting of salivary PRPs.

Salivary PRPs were electrophoretically separated on native gradient gels. The region of the gel containing the tannin-binding protein was cut out of the gel, minced into small pieces, and eluted from the gel into 4 vol of pH 8.8 Tris

(0.375 M). The eluate was dialyzed for 24 hr against water, and the dialysate was concentrated to 80 μ l using a Minicent-10 ultrafiltration device (BioRad Labs, Richmond, California 94804). A fraction of this material was evaluated for purity on an SDS gel, and the remainder was hydrolyzed in vacuo with 6 N Sequanal HCl (Pierce Chemical Co.) at 110°C for 24 hr. The hydrolyzate was dried under nitrogen and the amino acid analysis performed in duplicate by the Protein Structure Laboratory (Davis, California 95616).

The apparent molecular weight of the tannin-binding protein was determined by SDS gel electrophoresis. The sample was dissolved in equal volumes of solution A (1.6 ml glycerol, 0.4 g SDS, 2 mg bromophenol blue, and 8.4 ml water) and solution B (2 ml of 0.5 M Tris HCl, 1 ml of β -mercaptoethanol, and 7 ml water), heated in a boiling water bath for 4 min, and applied to the gel. Molecular weight standards were bovine serum albumin (66,000), egg albumin (45,000), trypsinogen (24,000), and β -lactoglobulin (18,400).

pH Dependence of PRP-Tannin Interactions. Purified condensed tannin from sorghum grain (Hagerman and Butler, 1980) was immobilized on epoxy-activated Sepharose (Sundberg and Porath, 1974). Suction-dried activated Sepharose (12 g) was mixed with 50 mg of the tannin, and 10 ml of 0.5 M Na_2CO_3 was added. The mixture was shaken gently at room temperature for 24 hr, and the gel was then washed as described by Armstrong (1976). Control affinity gel was prepared by reacting epoxy-activated Sepharose with 1 M ethanolamine instead of tannin. The affinity gels were stored at 4°C.

Small columns (5 ml) were prepared with the tannin and control affinity gels and were equilibrated at pH 8.0 (0.05 M Tris). Deer salivary PRPs (0.3 ml of a 4 mg/ml aqueous solution) were applied to the gels, which were then washed successively with 2-ml aliquots of pH 8.0, pH 6.0 (0.05 M phosphate), pH 4.0 (0.05 M acetate), and pH 2.0 (0.05 M glycine) buffers. The columns were then washed with 2 ml of 1% SDS. The eluate from each pH was collected and concentrated to a volume of about 30 μ l with a Minicent-10 ultrafiltration device. The concentrated samples were prepared for SDS gel electrophoresis as described above.

Survey of Saliva for Diet-Induced Changes in Protein Composition. Saliva samples were collected from resting esophageal-fistulated animals (two deer, two sheep) at various times during feeding trials and were immediately frozen. For analysis, samples were thawed, and a 40- μ l aliquot of each sample was mixed with 3 μ l of β -mercaptoethanol, 5 μ l of glycerol, and 5 μ l of solution A. The samples were then heated in a boiling water bath for 4 min, and 40 μ l of the mixture was applied to an SDS gel. After electrophoresis, the gels were stained and examined to determine whether new proteins were present in animals fed tannin-containing diets.

RESULTS AND DISCUSSION

Identification of Tannin-Binding Proteins. A method for identifying proteins with high affinity for tannins has been developed and applied to saliva from grazing and browsing ruminants. Seven major proteins are separated by native gel electrophoresis of deer saliva (Figure 1). When deer saliva is incu-

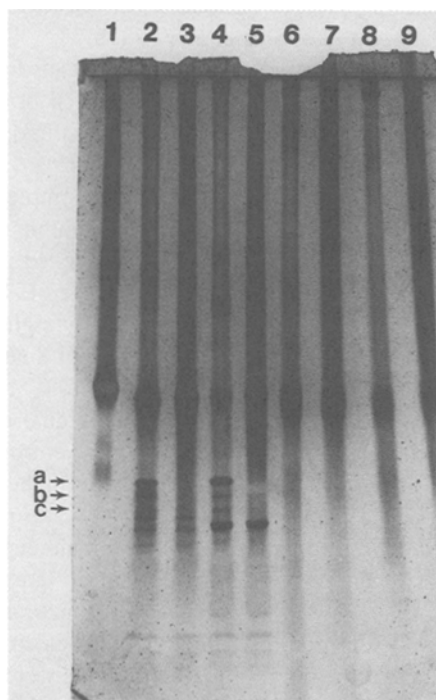


FIG. 1. Treatment of deer and sheep saliva with tannin. Saliva samples were mixed with tannin, incubated, and centrifuged before electrophoresis on a native gel. Proteins were fixed and stained with silver stain as described in the text. Tannin-binding proteins are indicated with arrows. The major tannin-binding protein, a, has an electrophoretic mobility of 0.64; b has a mobility of 0.66–0.67; c has a mobility of 0.69. Lane 1, bovine serum albumin (standard); lane 2, female deer saliva plus 50% methanol; lane 3, female deer saliva plus 0.8 μg tannic acid in 50% methanol; lane 4, male deer saliva plus 50% methanol; lane 5, male deer saliva plus 0.8 μg tannic acid in 50% methanol; lane 6, sheep (tannin-free diet) saliva plus 50% methanol; lane 7, sheep (tannin-free diet) saliva plus 0.8 μg tannic acid in 50% methanol; lane 8, sheep (tannin-containing diet) saliva plus 50% methanol; lane 9, sheep (tannin-containing diet) saliva plus 0.8 μg tannic acid in 50% methanol.

bated with a small amount of tannic acid before the electrophoresis, one major and two minor bands disappear, suggesting that the three proteins are bound to the tannin. The sheep saliva contains only one major protein band, which is unaltered by the addition of tannic acid to the sample (Figure 1). These results confirm earlier analyses of saliva from deer, sheep, and cattle in which a competitive binding assay was used to establish that deer saliva contains large amounts of tannin-binding protein but that sheep or cow saliva contains very little tannin-binding protein (Robbins et al., 1987b).

The electrophoretic method provides a simple means for surveying samples for tannin-binding proteins. The saliva is not purified or concentrated before electrophoresis. Very small amounts of tannin are required to detect the tannin-binding proteins. Both insoluble and soluble (Hagerman and Robbins, 1987) tannin-protein complexes are detected; insoluble complexes are centrifuged away from the sample before electrophoresis, and soluble complexes have different electrophoretic mobility than native, uncomplexed protein. The method is only useful for detecting complexes that form near pH 8, since the electrophoresis is run at pH 8.3. However, physiologically significant salivary protein-tannin interactions are expected to occur near pH 8 since the pH of saliva is between 7 and 9 (Robbins, 1983).

The major tannin-binding protein in two individual deer has an electrophoretic mobility of 0.64 (Figure 1). There is some heterogeneity in the minor tannin-binding proteins. For example, in the saliva from the female deer (Figure 1, lanes 2 and 3), the electrophoretic mobilities of the minor tannin-binding proteins are 0.66 and 0.67. In the saliva from the male deer (Figure 1, lanes 4 and 5), the mobilities of the minor tannin-binding proteins are 0.67 and 0.69. This heterogeneity may be due to differences in the size, amino acid composition, or glycosylation of the proteins. Proteins with electrophoretic mobilities similar to the mobilities of the tannin-binding proteins were also detected in the crude saliva of two other deer, but these proteins were not extensively characterized.

Isolation of Tannin-Binding Proteins. The tannin-binding salivary proteins of rodents are PRPs (Mehansho et al., 1983), and preliminary experiments with deer saliva suggested that ruminant tannin-binding proteins may also be proline rich (Robbins et al., 1987b). Deer saliva was treated with trichloroacetic acid, and the native gel electrophoresis assay for tannin-binding proteins was used to establish that the soluble material, which includes the PRPs (Mehansho et al., 1983), contained the tannin-binding proteins. The tannin-binding protein was further purified by electrophoresis. The isolated material contained one major protein and traces of a second, higher-molecular-weight protein (Figure 2). The major protein was retained by an ultrafiltration membrane with a molecular weight cutoff of 10,000 and had an apparent molecular weight of 19,000 by

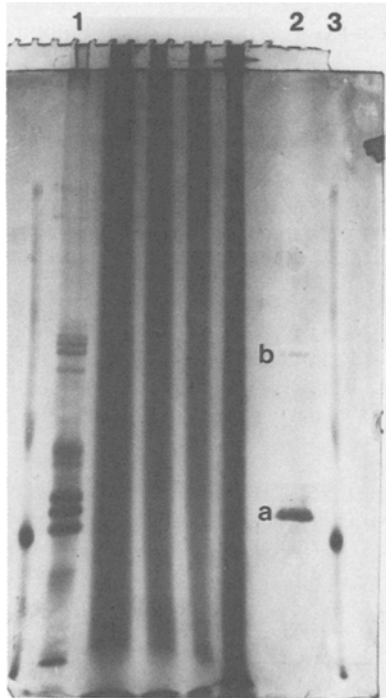


FIG. 2. Purification of tannin-binding protein from deer saliva. The tannin-binding protein was purified by trichloroacetic acid fractionation and native gel electrophoresis as described in the text. SDS gel electrophoresis was run to assess the purity of the isolated protein. The trichloroacetic acid-soluble fraction of the saliva is shown in lane 1; the purified protein is shown in lane 2. The major tannin-binding protein (a) and a minor tannin-binding protein (b) are indicated. Lane 3 contains molecular weight markers. The other lanes contain materials from an unrelated experiment.

SDS gel electrophoresis. Glucosamine and galactosamine were released from the tannin-binding protein upon acid hydrolysis, suggesting that the protein is glycosylated, like some PRPs from other mammals (Mehansho et al., 1985).

The tannin-binding protein from deer saliva contains larger amounts of proline, glutamate/glutamine, and glycine than of the other amino acids (Table 1). The protein does not contain any sulfur-containing amino acids and contains low amounts of the aromatic amino acids. The protein is thus superficially similar to the PRPs found in several mammals (Bennick, 1982; Mehansho et al., 1983, 1985). However, several amino acids, such as valine and isoleucine, which are not abundant in the PRPs of other mammals, were present in the deer tannin-binding protein. The differences in amino acid composition show that

TABLE 1. AMINO ACID COMPOSITION OF TANNIN-BINDING PROTEIN FROM DEER SALIVA^a

Amino acid	Mole percent		
	Deer tannin-binding protein	Rat PRP ^b	Mouse PRP ^c
Asx	8.5	2.5	1.9
Thr	6.0	—	3.6
Ser	8.3	1.4	1.4
Glx	13.4	24.0	18.4
Pro	19.7	41.3	45.6
Gly	14.4	24.2	18.2
Ala	5.3	—	1.6
Val	5.1	—	—
Ile	2.1	—	0.6
Leu	5.1	1.3	0.9
Tyr	1.3	—	—
Phe	2.6	—	—
Lys	3.9	0.6	—
His	1.7	0.2	—
Arg	2.8	4.5	7.0

^aThe protein was purified by trichloroacetic acid fractionation and electrophoresis as described in the text. The amino acid composition was determined after acid hydrolysis. Values shown are the average of two independent determinations. Tryptophan was not determined.

^bTannin-induced PRP described by Mehansho et al. (1983).

^cTannin-induced glycosylated PRP described by Mehansho et al. (1985).

the tannin-binding proteins from deer saliva are not identical to the PRPs found in saliva of nonruminant mammals. It is possible that salivary proteins of each animal are adapted to the particular tannin-containing foods consumed by that animal. Further studies of the amino acid composition and sequence of proteins from various animals are required to clarify the relationship between the tannin-binding protein of deer and PRPs of other mammals.

Interactions between Tannin-Binding Proteins and Tannin. The trichloroacetic acid-soluble, PRP-enriched fraction of deer saliva was used to characterize the interaction between the tannin-binding protein and various tannin samples. Electrophoretic analysis showed that the protein interacted with purified hydrolyzable or condensed tannin and with crude tannin from a variety of plants. The tannin-binding protein did not interact with plant extracts that had been treated with polyvinylpyrrolidone to adsorb the tannin and other polyphenolics (Loomis and Battaile, 1966), demonstrating that the interaction involved tannin and not some other protein-binding component of the extract.

Addition of polyvinylpyrrolidone to a mixture of tannin and the PRPs reversed the interaction between the salivary protein and tannin, suggesting that polyvinylpyrrolidone has a higher affinity for tannin than the salivary tannin-binding protein. The tannin-binding protein did not interact with tannin in the presence of SDS. Tannin-salivary protein complexes could be dissociated with SDS.

The complex of tannin and tannin-binding protein was soluble under all conditions examined. After incubating tannin with the PRP-enriched fraction of deer saliva, the mixture was centrifuged. No precipitate was visible. Part of the supernatant was electrophoresed on a native gel, and the remainder electrophoresed on an SDS gel. As expected, the tannin-binding protein had interacted with the tannin and could not be seen in the native gel. However, the tannin-binding protein was visible in the SDS gels. The SDS dissociated the tannin-protein complex so that the protein entered the gel and ran with its usual electrophoretic mobility. Thus, the tannin-binding protein was not part of an insoluble tannin-protein complex, which would have been pelleted during the centrifugation step. Viscosity data (S. Mole, Purdue University, unpublished) also suggests that proteins in deer saliva form soluble complexes with tannins. The tendency of the tannin-binding protein to form soluble complexes with tannin may be a consequence of glycosylation. Most proteins form insoluble complexes with tannin, except when large excesses of protein are present (Hagerman and Robbins, 1987; Mole and Waterman, 1987). However, glycoproteins tend to form soluble rather than insoluble complexes with tannin (Asquith et al., 1987).

The salivary protein has a high affinity for tannin. The native gel electrophoresis assay was used to compare the affinity of several proteins for tannin. In the presence of a 40-fold weight excess (10-fold molar excess) of bovine serum albumin or a fivefold weight excess (equimolar) of ribulose biphosphate carboxylase/oxygenase, the tannin preferentially bound the salivary protein. The high affinity of the salivary protein for tannin suggests that, during the chewing/rumination process, dietary tannin may interact with the salivary tannin-binding protein. Other proteins, including dietary proteins, may not complex any tannin in the presence of the salivary tannin-binding protein.

Tannin-protein interactions are pH dependent (Hagerman and Butler, 1978). For example, bovine serum albumin is precipitated by tannin only at pH values near 5, while ribulose biphosphate carboxylase/oxygenase is precipitated between pH 6 and pH 7 (Martin and Martin, 1983). The gastrointestinal tract of ruminants has regions of pH varying from pH 2 (abomasum) to pH 9 (saliva) (Robbins, 1983). If salivary tannin-binding proteins protect other proteins from tannin at all stages of the digestive process, the salivary proteins must form complexes that are stable over this range of pH values. Since the tannin-binding protein forms soluble rather than precipitable complexes with tannin, conventional precipitation assays could not be used to examine the pH

dependence of tannin-salivary protein complex formation. Instead, condensed tannin was immobilized on an insoluble matrix, and the binding of salivary proteins to the gel was examined as a function of pH.

In preliminary experiments, the immobilized tannin bound proteins in a pH-dependent fashion; for example, bovine serum albumin was bound to the derivatized matrix only at pH values near 5 (Suchar, 1987). The control matrix that was not derivatized with tannin did not bind protein. The deer salivary tannin-binding protein was bound to the derivitized gel at pH 8 and was not washed off the gel by sequential treatment with solutions of pH 6, pH 4, or pH 2. The tannin-binding protein and two minor higher-molecular-weight salivary proteins were recovered from the gel in an SDS wash that followed the pH 2 wash. One of the higher-molecular-weight proteins is the minor tannin-binding protein that copurified with the major tannin-binding protein in other experiments (Figure 2, band b). The remaining salivary proteins were not bound by the gel or were washed from the gel with the pH 6 wash. These results suggest that the tannin-binding protein could bind dietary tannin in the mouth of the deer and that the complex would be stable at pH values encountered during transit of the entire digestive tract. We are currently investigating the stability of the complexes in the presence of proteases, bile salts, and other constituents of the digestive tract that may disrupt tannin-protein complexes (Martin et al., 1987).

Induced Synthesis of Tannin-Binding Proteins. In some rodents, synthesis of PRPs is induced by dietary tannins (Mehansho et al., 1983, 1985). To determine whether synthesis of salivary tannin-binding proteins was induced in deer or sheep, saliva was collected at intervals during a feeding trial. The saliva was examined with SDS gel electrophoresis, which resolves more proteins and is more sensitive than native gel electrophoresis. No new proteins were detected in the saliva of two individual deer (Figure 3A) during a 10-day feeding trial with diets containing 2% tannic acid. These results confirm the results of previous analyses of the saliva from animals on this feeding trial, in which neither crude nitrogen nor tannin-binding capacity changed during the experimental period (Robbins et al., 1987b). The deer used in this experiment were adult animals and had been exposed to dietary tannin in several earlier experiments. It seems likely that these animals have already adapted to dietary tannin and produce salivary tannin-binding proteins continuously. In experiments with deer not previously exposed to tannin, it might be possible to detect induction of synthesis of salivary tannin-binding proteins analogous to the induction observed in some rodents.

No new proteins were observed in the saliva collected from two individual sheep during the feeding trial with 2% tannic acid (Figure 3B). There are only about 12 proteins in the sheep saliva, while there are at least 20 proteins in the deer saliva. The sheep had never been exposed to dietary tannin before this

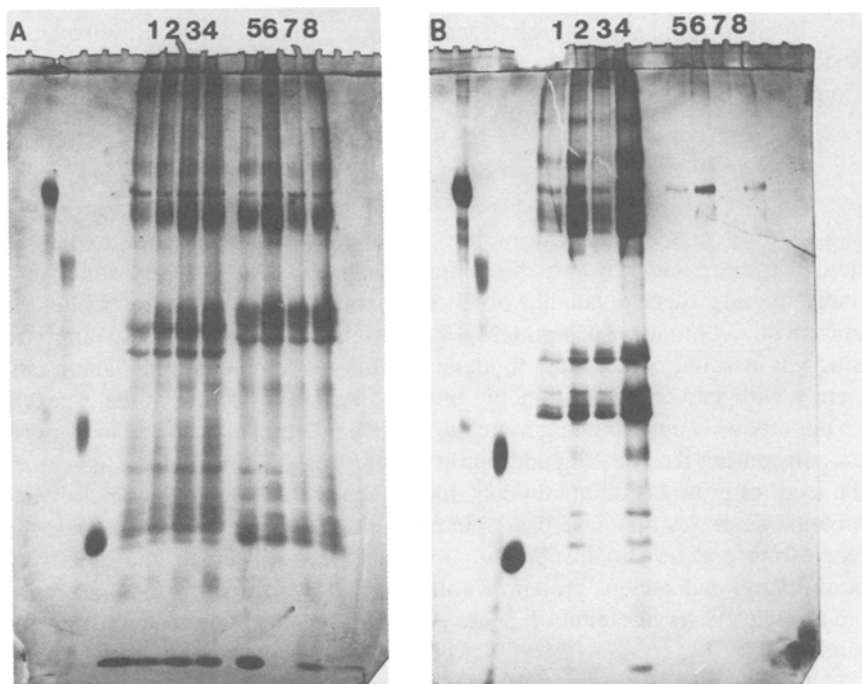


FIG. 3. Saliva composition during a feeding trial. Saliva samples were taken from animals before (day -1) and on the third, sixth, and tenth day of a feeding trial in which 2% tannic acid was incorporated into the diet. The cows were not given tannin-containing diets. The samples were electrophoresed on SDS gels as described in the text. Four lanes on left of each gel contain molecular weight markers. (A) Lane 1, female deer, day -1; lane 2, female deer, day 3; lane 3, female deer, day 6; lane 4, female deer, day 10; lane 5, male deer, day -1; lane 6, male deer, day 3; lane 7, male deer, day 6; lane 8, male deer, day 10. (B) Lane 1, sheep, day -1; lane 2, sheep, day 3; lane 3, sheep, day 6; lane 4, sheep, day 10; lane 5, cow NMSU, #2507; lane 6, cow NMSU, #2509; lane 7, cow, horned Hereford #2; lane 8, cow, polled Hereford #10.

feeding trial, so the absence of induced salivary tannin-binding proteins suggests that these animals, like hamsters (Mehansho et al., 1987), do not produce tannin-binding salivary proteins in response to tannin-containing diets. Alternatively, it might be argued that longer exposure times, higher levels of dietary tannin, or a different source of dietary tannin may be necessary for induction of protein synthesis in sheep.

Although cattle were not fed tannins, saliva from four cattle was analyzed electrophoretically (Figure 3B). Only one protein was detected in the cow saliva, in agreement with its low nitrogen content (Robbins et al., 1987b).

In all of these studies, saliva was collected from esophageally fistulated, resting animals. We plan to evaluate saliva collected from chewing animals and from individual salivary glands in future studies.

CONCLUSIONS

The saliva of deer, a browsing animal, has been compared to that of domestic sheep and cow, which are grazing animals. The browser, which normally ingests dietary tannin, produces tannin-binding proteins, while the grazers do not produce such proteins. We postulate that browsers may minimize nitrogen loss and toxicity due to dietary tannin by complexing the tannin efficiently with salivary tannin-binding proteins. According to our model, grazers do not produce tannin-binding proteins, so dietary tannin may be bound by other proteins during the chewing and rumination process. If this hypothesis is true, the feces of browsers on tannin-containing diets should contain tannin-salivary protein complexes that have passed intact through the digestive tract, while the feces from grazers should contain complexes between tannin and dietary or nonsalivary, endogenous protein. Furthermore, grazers may be more susceptible to toxic effects of tannins than are browsers, which are protected by tannin-binding proteins.

A method has been developed for detecting tannin-binding proteins in the saliva of herbivores. The method is simple and requires only small quantities of crude saliva. To establish whether other grazers and browsers follow the pattern observed with deer, sheep, and cows, saliva from a variety of other grazing and browsing ruminants should be analyzed. In addition, the method could be used to screen nonruminants for salivary tannin-binding proteins that may provide an effective defense against this common plant allelochemical.

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IDENTIFICATION AND FIELD TESTING OF
ADDITIONAL COMPONENTS OF FEMALE
SEX PHEROMONE OF AFRICAN
ARMYWORM, *Spodoptera exempta*
(LEPIDOPTERA: NOCTUIDAE)

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Abstract—Ovipositor washings from virgin female *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae) were analyzed by high-resolution gas chromatography (GC) linked to a male electroantennogram (EAG). GC retention times of the two major EAG responses observed were consistent with their assignment as (Z)-9-tetradecenyl acetate and (Z,E)-9,12-tetradecadienyl acetate, as previously identified. However, three other EAG responses were also noted that had GC retention times consistent with (Z)-9-tetradecenal, (Z)-9-tetradecen-1-ol, and (Z)-11-hexadecenyl acetate. The components were present in the ratio of 100:5:1.5:3.5:4, respectively. Further analysis of the ovipositor washings by GC linked to a mass spectrometer (GC-MS) confirmed these findings and indicated the presence of a sixth component consistent with (Z)-11-tetradecenyl acetate present at 2% of the major component. In field tests carried out in Kenya, (Z)-11-hexadecenyl acetate was the only newly identified component to enhance the catch of the original two-component mixture when presented in their natural ratio. The addition of (Z)-9-tetradecen-1-ol reduced catch, while (Z)-9-tetradecenal and (Z)-11-tetradecenyl acetate had no apparent effect.

Key Words—*Spodoptera exempta*, Lepidoptera, Noctuidae, sex pheromone, (Z)-9-tetradecenyl acetate, (Z)-9-tetradecenyl acetate, (Z)-11-tetradecenyl acetate, (Z,E)-9,12-tetradecadienyl acetate, (Z)-9-tetradecen-1-ol, (Z)-11-hexadecenyl acetate.

INTRODUCTION

The African armyworm, *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae), is a serious but sporadic pest of graminaceous crops, including forage grasses, maize, wheat, rice, and millet, throughout Africa. It is also known to occur in Southeast Asia, Philippines, New Guinea, and Australia (Haggis, 1986). Beevor et al. (1975) identified two components of the female sex pheromone as (Z)-9-tetradecenyl acetate and (Z,E)-9,12-tetradecadienyl acetate in the ratio of 100:5. These were field tested in Kenya by Campion et al. (1976) and found to be highly attractive to male *S. exempta*, although the catches were significantly less than those with a light trap.

S. exempta has been shown to be a migratory moth from both indirect (Brown et al., 1969) and direct evidence (Rose and Dewhurst, 1979; Riley et al., 1983; Rose et al., 1985). By tracking their progress using pheromone and light traps over many years and relating these data to weather movements, it is now possible to forecast when and where outbreaks of armyworm larvae may occur (Haggis, 1986).

However, outbreaks do not take place throughout the year but appear to be seasonally restricted to coincide with rain-bearing wind movements, such as the intertropical convergence zone (Tucker and Pedgley, 1983; Haggis, 1986). In most years, there is a gap of two to four months after the rainy season in which few, if any, outbreaks are reported (Haggis, 1986). There do not appear to be major return migrations towards the equatorial region where the first seasonal outbreaks are recorded, and low density, nonmigrating populations of insects are thought to survive until more favorable conditions permit further population growth (Rose, 1979; Nyirenda, 1985). In order to study areas with low population levels, it is important to maximize the ability of the pheromone trap to attract and catch males of the target species. This is particularly important when closely related sympatric species, such as *Spodoptera triturrata* are present and known to be attracted to a similar pheromone (Blair and Tannock, 1977; D.J. Chamberlain, personal communication). As part of this process, this paper describes a reexamination of the sex pheromone derived from adults of the gregarious-phase larvae (Faure, 1943) and the preliminary field evaluation of the new compounds found.

METHODS AND MATERIALS

Insect Material. In order to carry out chemical and electrophysiological analyses, sixth-instar larvae were field collected in Kenya, reared on fresh grass, and allowed to pupate in bowls containing dry sandy soil. Pupae were dispatched by air to London, where they were sexed and maintained in an environmental cabinet on a reversed 12-hr light, 12-hr dark cycle, with temperatures alternating from 25°C to 22°C and a relative humidity of 70%. Adult moths were highly excitable and so were maintained with a 10% sucrose solution in groups of no more than 10 per container (Perspex, 28 × 15 × 9 cm) with tissue paper to discourage flying.

Pheromone Collection. Ovipositor washings were prepared in hexane as described by Sower et al. (1973), using virgin female moths between 1 and 4 days old and usually between 8 and 9 hr into the dark phase. Volatiles from 2- to 3-day old virgin female moths were collected on charcoal filters (5 mg) as previously described (Grob and Zurcher, 1976; Nesbitt et al., 1979; Tumlinson et al., 1982).

Gas Chromatography (GC). GC analyses were conducted on a Carlo Erba Fractovap 4160 instrument fitted with a Grob split/splitless injector (220°C) and flame ionization detector (220°C). Fused silica capillary columns (25 m × 0.32 mm ID) were used coated with polar CP Wax 57CB (chemically bonded Carbowax 20 M; 0.21 μm film thickness; Chrompack), highly polar OV275 (dicyanoallyl silicone; 0.2 μm film thickness; Chrompack), or nonpolar CP Sil 5CB (chemically bonded methyl silicone; 0.12 μm film thickness; Chrompack). Carrier gas was helium (linear velocity 40 cm/sec). All injections were made with the split valve closed for 40 sec onto a column held at 70°C for 2 min, then temperature programmed as indicated in the tables. Retention times of the compounds identified in the ovipositor washings, entrainments, and their synthetic analogs are summarized in Table 1. Temperature-programmed conditions were used that allowed the retention times to be converted to equivalent chain lengths (ECLs) relative to the retention times of straight-chain acetates; thus, for example, tetradecyl acetate = 14.00 (Harris and Habgood, 1966). Retention times of series of geometrical isomers and the natural compounds were usually obtained under isothermal conditions, so their retention times are quoted in minutes.

Mass Spectrometry (GC-MS). Electron impact mass spectra were obtained on a Finnigan ITD 700 with open split interface to a Carlo Erba 5130 GC fitted with split/splitless injector (220°C) and fused silica capillary column (25 m × 0.32 mm ID) coated with BP20 (chemically bonded Carbowax 20 M; 0.2 μm film thickness; S.G.E.). Carrier gas was helium (23 cm/sec). The oven tem-

TABLE 1. GC RETENTION TIMES OF PHEROMONE COMPONENTS OF *Spodoptera exempta* AND THEIR RELATIVE ABUNDANCE IN OVIPOSITOR WASHINGS

Compound	Retention times (ECLs) ^a		Relative composition
	CP Wax 57CB ^b	CP Sil 5CB ^b	
I Z9-14:Ald	12.82	11.81	1.5
II Z9-14:Ac	14.27	13.83	100
III Z11-14:Ac	14.43	13.95	2
IV Z, E9, 12-14:Ac	15.01	13.91	5
V Z9-14:OH	15.16	12.52	3.5
VI Z11-16:Ac	16.27	15.83	4

^aRetention times in equivalent chain length units relative to the retention times of straight chain acetates.

^bOven temperature 70°C for 2 min then programmed at 20°C/min to 120°C, and then at 4°C/min to 210°C.

perature was held at 70°C for 2 min, then programmed to 120°C at 20°C/min and then at 4°C/min to 220°C.

Electroantennography (EAG). Simultaneous recording of EAG responses from male antennae to GC column effluent were conducted essentially as described by Beevor et al. (1986), except that the indifferent microelectrode was inserted into an interstitial membrane at the proximal end of one flagellum and the recording electrode inserted into an interstitial membrane at the distal end of the other flagellum. Eluting compounds were deemed to have caused an EAG response if the amplitude of the response was at least 10% greater than the average of responses to fractions eluting before and after it. GC columns and operating conditions were as described above. EAG responses to synthetic compounds were tested by pulsing nitrogen through a Pasteur pipet containing 5 ng of the test compound applied in 2 μ l of hexane, under conditions described by Beevor et al. (1986).

Synthetic Chemicals. (*Z,E*)-9,12-Tetradecadienyl acetate was obtained from Food Industries Ltd. (U.K.) in 1978 and contained 99.4% *Z,E*, <0.1% *E,Z*, 0.2% *Z,Z*, and 0.36% *E,E*. The *E,Z*, *E,E*, and *Z,Z* isomers of 9,12-tetradecadienyl acetate were obtained from the Institute of Pesticide Research, Wageningen, The Netherlands.

Wittig reaction with potassium *tert*-butoxide in THF between the triphenylphosphonium salt of 9-bromo-1-nonanol and (*E*)-2-pentenal gave a mixture of (*Z,E*)- and (*E,E*)-9,11-tetradecadienyl acetate. Similar reaction between the triphenylphosphonium salt of 8-bromo-1-octanol and (*E*)-2-hexenal gave the isomers of 8,10-tetradecadienyl acetate. These conjugated dienes could be iso-

merized to the thermodynamic equilibrium mixture of isomers by exposure to sunlight of a hexane solution containing a catalytic amount of iodine (cf. Beevor et al., 1986). Monounsaturated alcohols, acetates, and aldehydes were obtained by standard Wittig or acetylenic routes. The *Z* isomers used in field tests contained no more than 2% of the corresponding *E* isomers.

Field Tests. Field trials of synthetic pheromone mixtures were conducted in a mixture of cotton, maize, and fallow fields at the Cotton Research Station (Kibos, Kenya) in 1986 and 1987. Pheromone dispensers were white rubber septa (Aldrich, catalog No. Z10,072-2) impregnated with 0.1 ml of a hexane solution containing 1 mg of the pheromone blend and an equal weight of 2,6-*tert*-butyl-4-methylphenol (BHT) as antioxidant.

In 1986, sticky disk traps (60 cm diameter; Biological Control Systems Ltd., Treforest, Mid Glamorgan, U.K.) were used, fastened to a wooden T-shaped support 1.5 m above ground level. The pheromone dispenser was suspended at the center beneath a small inverted V-shaped roof to protect it from direct sunlight. Subsequent field work was conducted with funnel traps (8 cm diameter yellow funnel with white collecting box and green lid 3 cm above funnel rim; Biological Control Systems Ltd., U.K.), which were found to be as effective as disk traps and easier to maintain (Murlis et al., unpublished results). Each trap was fastened to an inverted L-shaped metal support 1.5 m from ground level. Lures were not changed during a trial. The traps were placed in circles containing one replicate of each treatment with at least 25 m between nearest neighbors and at least 100 m between replicates. Treatments were randomly assigned to each trap within a replicate and moved on one position clockwise each day, at which time the trap catch was recorded and discarded. The mean nightly catches were transformed to $\log(x + 1)$ to stabilize the variance, before subjecting them to analysis of variance. Differences between means were tested for significance at the 5% level by Duncan's multiple-range test (DMRT).

RESULTS AND DISCUSSION

Structure Determination. Initial analyses of ovipositor washings by linked GC-EAG on polar CP Wax 57CB and OV-275 and nonpolar CP Sil 5CB columns confirmed the presence of (*Z*)-9-tetradecenyl acetate (Z9-14:Ac) and (*Z,E*)-9,12-tetradecadienyl acetate (*Z,E*9,12-14:Ac) in the ratio of 20:1 as the major EAG active compounds, as described by Beevor et al. (1975). Pheromone yields of up to 200 ng of Z9-14:Ac were obtained from the ovipositor washings, but 20 ng was more usual. The reasons for this difference were not investigated. Increasing the quantity of extract tested (equivalent to 80 ng of Z9-14:Ac) and using temperature programmed runs to cover the range of reten-

tion times typical of lepidopteran pheromone components between decyl and eicosyl acetates, it became apparent that there were three additional EAG active compounds that had not been previously observed. Each of these compounds constituted 5% or less of the major component, Z9-14:Ac, by GC peak height.

Relative GC retention times of these compounds in ECLs are tabulated in Table 1 together with those of a sixth compound detected by GC-MS and identified by comparison of GC retention times with synthetic analogs. For convenience, the compounds are designated I-VI on the basis of their elution order on a polar CP Wax 57CB column.

Compound I displayed GC retention times characteristic of either a dodecyl acetate or a tetradecenal. The absence of ions at m/z 61 ($\text{Me}\cdot\text{COOH}^+$) and m/z 166 (M-60) (Figure 1) from the MS, which are indicative of a dodecyl acetate (Buser and Arn, 1975; Löfstedt et al., 1982), suggested it was a tetradecenal isomer. This was supported by the presence of an ion at m/z 192, which could have arisen through the loss of water from a tetradecenal (Gudziniowicz et al., 1976) or a loss of CH_3COOH from a tetradecadienyl acetate. The latter would be excluded on the basis of retention time. The position and geometry of the double bond were established as the (Z)-9 isomer by comparison of GC retention times with a range of synthetic analogs on polar CP Wax 57CB and nonpolar CP Sil 5CB columns (Table 2).

The GC retention times (Table 1) and MS [m/z 61, 194 (M-60)] (Figure 1) of compounds II and III indicated they were tetradecenyl acetate isomers. Comparison of their relative retention times with the complete range of tetradecenyl acetate isomers on polar CP Wax 57CB and the nonpolar CP Sil 5CB column with (Z)-12-tetradecenyl acetate as an internal standard (Nesbitt et al., 1986) showed that retention times of compound II were consistent only with those of Z9-14:Ac, as found by Beevor et al. (1975), and retention times of compound III were consistent only with those of (Z)-11-tetradecenyl acetate (Z11-14:Ac). No EAG response was recorded at any time to Z11-14:Ac, presumably because it eluted just after the two major EAG-active compounds in the extract, namely, Z9-14:Ac on the polar columns and Z,E9,12-14:Ac (compound IV) on the nonpolar. Linked GC-EAG analyses with synthetic mixtures of these compounds in the same relative amounts also failed to yield a response to Z11-14:Ac.

The MS of compound IV was characteristic of a tetradecadienyl acetate (M-60 at m/z 192; m/z 61) (Figure 1), and comparison of GC retention times of the isomers of 9,12-, 9,11-, and 8,10-tetradecadienyl acetates on polar CP Wax 57CB and nonpolar CP Sil 5CB columns confirmed the original assignment of Z,E9,12-14:Ac (Beevor et al., 1975) (Table 3).

Compound V had GC retention times (Table 1) and MS (M-18 at m/z 194; no m/z 61) (Figure 1) consistent with a tetradecen-1-ol isomer. Comparison of its GC retention times with those of a range of synthetic tetradecen-1-

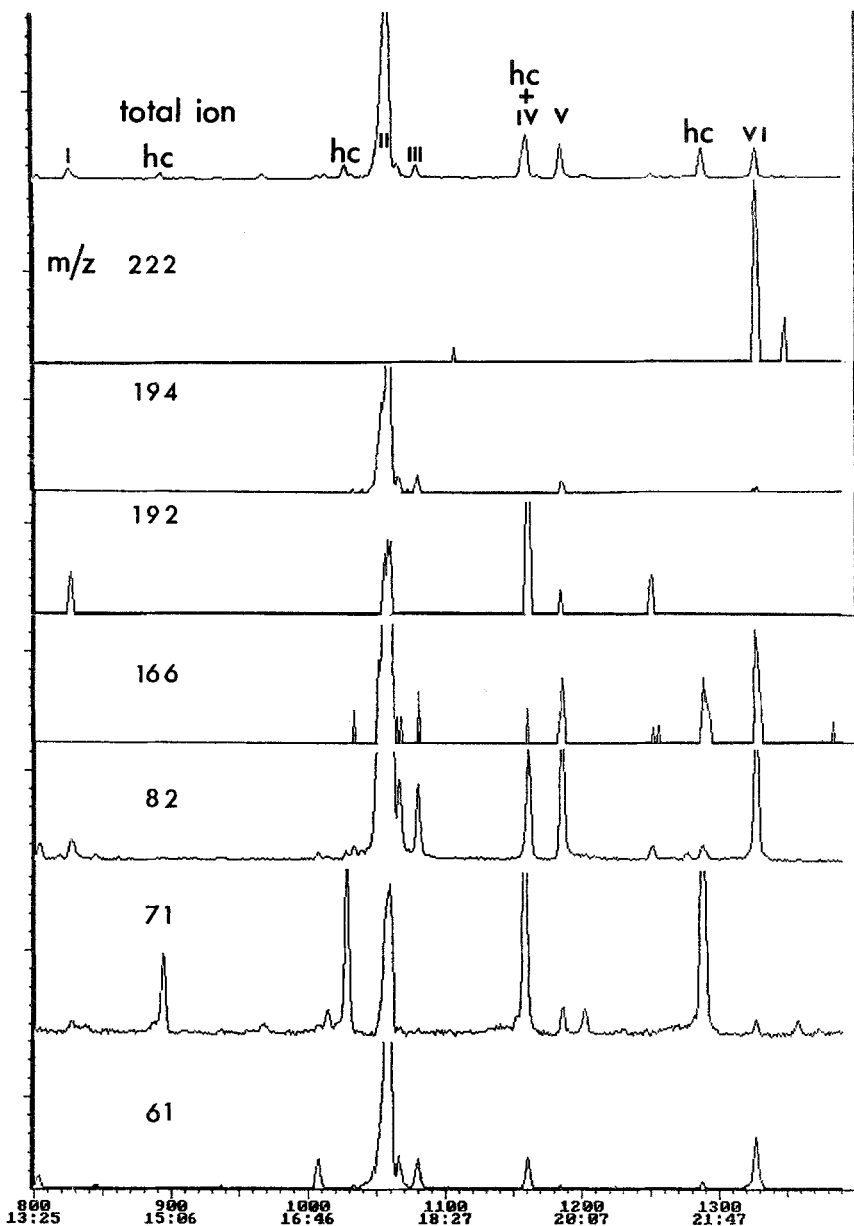


FIG. 1. Total ion chromatogram, m/z 40–300, and mass chromatograms of m/z 61, 71, 82, 166, 192, 194, and 222 of an extract corresponding to five female *S. exempta* abdominal tips. m/z 61 is an ion typical of acetates, while m/z 71 is typical of saturated hydrocarbons, and m/z 82 is typical of unsaturated straight-chain compounds. Ions at m/z 166, 194, and 222 are produced by loss of CH_3COOH from dodecanyl, tetradecanyl, and hexadecanyl acetates, respectively, or loss of H_2O from the corresponding alcohols. Similarly, ions at m/z 192 are characteristic of the loss of CH_3COOH from tetradecadienyl acetates or H_2O from tetradecenals and tetradecadienols. Phomone components are labeled as in the text. Straight-chain hydrocarbons are labeled hc.

TABLE 2. RETENTION TIMES OF SYNTHETIC TETRADECENALS AND COMPONENT I

Compound	Retention times (min)	
	CP Wax 57CB ^a	CP Sil 5CB ^a
Component I	22.41	20.02
14: Ald	21.47	20.28
13-14: Ald	22.87	20.25
Z12-14: Ald	23.67	20.91
E12-14: Ald	23.14	20.58
Z11-14: Ald	22.88	20.41
E11-14: Ald	22.58	20.32
Z10-14: Ald	22.60	20.21
E10-14: Ald	22.31	20.20
Z9-14: Ald	22.41	20.02
E9-14: Ald	22.27	20.06
Z8-14: Ald	22.24	19.94
E8-14: Ald	22.19	19.99
Z7-14: Ald	22.20	19.83
E7-14: Ald	22.12	20.02
Z6-14: Ald	22.19	19.88
E6-14: Ald	22.13	20.00
Z5-14: Ald	21.97	19.83
E5-14: Ald	22.02	20.00
Z4-14: Ald	22.27	19.97

^aOven temperature 70°C for 2 min then programmed at 4°C/min to 220°C.

o1 isomers under isothermal conditions on both polar and nonpolar columns, using (*Z*)-12-tetradecen-1-ol as an internal standard, showed them to be consistent only with those of (*Z*)-9-tetradecen-1-ol (*Z*9-14:OH) (Table 4).

GC retention times (Table 1) and MS [m/z 61, 222 ($M-60$)] (Figure 1) of compound VI were consistent with those of a hexadecenyl acetate. All the hexadecenyl acetate isomers were available for comparison of their GC retention times with those of compound VI except (*Z*)- and (*E*)-2-hexadecenyl acetates. However, by analogy with the GC retention times of tetradecenyl acetate isomers, which follow a similar pattern (Nesbitt et al., 1986), (*Z*)- and (*E*)-2-hexadecenyl acetates could be excluded as candidates for compound VI. The GC retention times of compound VI were consistent only with those of (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) on all three columns used (Table 5).

TABLE 3. RETENTION TIMES OF SYNTHETIC TETRADECADIENYL ACETATES AND COMPONENT IV

Compound	Retention times (min)	
	CP Wax 57CB ^a	CP Sil 5CB ^b
Component IV	28.50	26.10
<i>Z,E</i> 9,12-14: Ac	28.44	26.08
<i>E,Z</i> 9,12-14: Ac	28.91	26.70
<i>Z,Z</i> 9,12-14: Ac	28.91	27.20
<i>E,E</i> 9,12-14: Ac	28.44	26.29
<i>Z,E</i> 8,10-14: Ac	29.75	29.13
<i>E,Z</i> 8,10-14: Ac	30.19	30.10
<i>Z,Z</i> 8,10-14: Ac	30.38	31.40
<i>E,E</i> 8,10-14: Ac	30.58	32.40
<i>Z,E</i> 9,11-14: Ac	30.05	29.95
<i>E,Z</i> 9,11-14: Ac	30.32	31.17
<i>Z,Z</i> 9,11-14: Ac	30.47	31.88
<i>E,E</i> 9,11-14: Ac	30.79	32.85

^aOven temperature 70°C for 2 min then programmed at 4°C/min to 220°C.

^bOven temperature 70°C for 2 min then programmed at 20°C/min to 130°C.

Analysis of entrained volatiles from virgin females by GC-EAG and GC-MS indicated that the pheromone released by the female was similar to that found in ovipositor extracts. Yields of up to 50 ng/female/night of the major component *Z*9-14: Ac were collected. To ensure the entrainment apparatus was functioning, a synthetic mixture of 100 ng of each compound except *Z*11-14: Ac was placed on aluminium foil. Recovery was between 50 and 80% for each compound in 4 hr.

The relative EAG responses of the six compounds identified in the female ovipositor washings at the 5-ng level were: I, 0.77 mV; II, 1.63 mV; III, 1.19 mV; IV, 2.46 mV; V, 1.0 mV; VI, 0.83 mV; and solvent blank, 0.6 mV.

GC-MS also confirmed the presence of a complete series of straight-chain saturated hydrocarbons (HC) in varying amounts from 21 HC to 29 HC, in the extracts (Figure 1). No EAG responses were ever observed to these compounds in linked GC-EAG analyses.

Field Tests. In order to assess the effect of individual compounds on male attractancy to pheromone traps, field trials conducted in 1986 compared the total mixture of six compounds, in their naturally occurring ratio, with five-component mixtures in which either *Z*9-14: Ald, *Z*9-14: OH, *Z*11-14: Ac, or *Z*11-

TABLE 4. GC RETENTION TIMES OF SYNTHETIC TETRADECEN-1-OLS AND COMPONENT V

Compound	Retention times (min)	
	CP Wax 57CB ^a	CP Sil 5CB ^b
Component V	32.34	27.75
14:OH	28.33	30.23
13-14:OH	34.31	28.73
Z12-14:OH	38.44	32.02
E12-14:OH	35.77	30.51
Z11-14:OH	34.36	29.48
E11-14:OH	33.30	29.13
Z10-14:OH	33.28	28.66
E10-14:OH	32.02	28.57
Z9-14:OH	32.30	27.75
E9-14:OH	31.82	28.13
Z8-14:OH	31.82	27.30
E8-14:OH	31.35	27.77
Z7-14:OH	31.31	26.95
E7-14:OH	31.08	27.66
Z6-14:OH	31.30	27.00
E6-14:OH	31.04	27.47
Z5-14:OH	31.73	27.32
E5-14:OH	31.59	27.89
Z4-14:OH	30.70	27.05
E4-14:OH	30.70	27.80
Z3-14:OH	30.21	27.22

^aOven temperature 70°C for 2 min then programmed at 20°C/min to 135°C.

^bOven temperature 70°C for 2 min then programmed at 20°C/min to 115°C.

16:Ac had been removed. Z9-14:Ac and Z,E9,12-14:Ac were present in all lures tested because they were assumed to be necessary for attraction.

Removal of either Z9-14:Ald or Z11-14:Ac was found to have no effect on trap catch compared to the total mixture. However, removing Z11-16:Ac significantly reduced catch and removing Z9-14:OH significantly increased catch (Table 6).

The two-component mixture of Z9-14:Ac and Z,E9,12-14:Ac in their naturally occurring ratio of approximately 100:5, caught significantly more males than the six-component mixture, although it was significantly less attrac-

TABLE 5. GC RETENTION TIMES OF SYNTHETIC HEXADECENYL ACETATES AND COMPONENT VI

Compound	Retention times (min)		
	CP Wax 57CB ^a	OV 275 ^a	CP Sil 5 CB ^b
Component VI	29.39	21.67	30.90
16:Ac	27.12	20.70	31.28
15-16:Ac	31.55	23.02	31.03
Z14-16:Ac	35.00	25.00	31.62
E14-16:Ac	32.78	23.30	31.28
Z13-16:Ac	31.40	22.80	31.18
E13-16:Ac	30.38	21.80	31.05
Z12-16:Ac	30.35	22.25	31.04
E12-16:Ac	29.50	21.35	30.99
Z11-16:Ac	29.38	21.65	30.84
E11-16:Ac	29.20	21.20	30.91
Z10-16:Ac	28.75	21.10	30.72
E10-16:Ac	28.65	20.82	30.82
Z9-16:Ac	28.13	20.75	30.68
E9-16:Ac	28.30	20.55	30.76
Z8-16:Ac	27.85	20.55	30.52
E8-16:Ac	28.02	20.30	30.68
Z7-16:Ac	27.47	20.40	30.50
E7-16:Ac	27.88	20.15	30.68
Z6-16:Ac	27.25	20.20	30.44
E6-16:Ac	27.76	20.15	30.70
Z5-16:Ac	27.45	20.23	30.53
E5-16:Ac	27.90	20.15	30.69
Z4-16:Ac	26.63	20.00	30.49
E4-16:Ac	27.88	20.00	30.77
Z3-16:Ac	27.35	19.90	30.64
E3-16:Ac	27.60	19.65	30.72

^aOven temperature 70°C for 2 min then programmed at 20°C/min to 120°C.

^bOven temperature 70°C for 2 min then programmed at 4°C/min to 210°C.

tive than the five-component mixture in which Z9-14:OH had been removed. For comparative purposes, the "standard" lure used throughout East Africa, (Nyirenda, 1985, Haggis, 1986), a 100:7.5 mixture of Z9-14:Ac and Z,E9,12-14:Ac, was also included in the trial. Catches with this were not significantly

TABLE 6. CATCHES OF MALE *S. exempta* MOTHS IN TRAPS BAITED WITH COMBINATIONS OF PHEROMONE COMPONENTS IN NATURALLY OCCURRING RATIOS AND STANDARD LURE OF 100:7.5, Z9-14:Ac to Z,E9,12-14:Ac (4 REPLICATES, 28 NIGHTS)

Pheromone component (μg)						Mean catch/ trap/night ^a
Z9-14:Ald	Z9-14:Ac	Z11-14:Ac	Z,E9,12-14:Ac	Z9-14:OH	Z11-16:Ac	
15	1000	20	50	35	50	22c
0	1000	20	50	35	50	18cd
15	1000	0	50	35	50	17cd
15	1000	20	50	0	50	51a
15	1000	20	50	35	0	15d
0	1000	0	50	0	0	40b
0	1000	0	75	0	0	46ab

^a Actual mean catches/trap/night; means followed by the same letter are not significantly different at the 5% level by DMRT using $\log(x + 1)$ transformed data.

different from those of either the two-component mixture with the natural ratio of compounds or the five-component mixture without Z9-14:OH, although the latter did catch 10% more moths (Table 6).

Because of the antagonistic nature of Z9-14:OH, it was not included in field trials conducted in 1987. Table 7 shows the results from a trial in which individual components were removed from the resulting five-component mixture. The most dramatic result was obtained by the removal of Z,E9,12-14:Ac, which resulted in no catch at all. Differences between catches for the other

TABLE 7. CATCHES OF MALE *S. exempta* MOTHS IN TRAPS BAITED WITH COMBINATIONS OF FIVE OF THE COMPOUNDS IDENTIFIED COMPARED TO TWO-COMPONENT MIXTURE (4 REPLICATES, 24 NIGHTS)

Pheromone component (μg)					Mean catch/ trap/night ^a
Z9-14:Ald	Z9-14:Ac	Z11-14:Ac	Z,E9,12-14:Ac	Z11-16:Ac	
15	1000	20	50	50	11.3a
15	1000	20	50	0	10.5a
15	1000	20	0	50	0b
15	1000	0	50	50	13.1a
0	1000	20	50	50	12.7a
0	1000	0	50	0	9.8a

^a Actual mean catches/trap/night; means followed by the same letter are not significantly different at the 5% level by DMRT using $\log(x + 1)$ transformed data.

TABLE 8. CATCHES OF MALE *S. exempta* MOTHS IN TRAPS BAITED WITH TWO- AND FIVE-COMPONENT BLENDS WITH DIFFERENT LEVELS OF Z,E9,12-14:Ac (4 REPLICATES, 24 NIGHTS)

Pheromone component (μg)					Mean catch/trap/night ^a
Z9-14:Ald	Z9-14:Ac	Z11-14:Ac	Z,E9,12-14:Ac	Z11-16:Ac	
15	1000	20	50	50	15.3a
15	1000	20	75	50	15.5a
15	1000	20	100	50	9.8b
0	1000	0	50	0	12.8b
0	1000	0	75	0	12.8b
0	1000	0	100	0	6.7c

^aActual mean catch/trap/night; means followed by the same letter are not significantly different at the 5% level by DMRT using $\log(x + 1)$ transformed data.

treatments were not significant, although in each case mixtures without Z11-16:Ac caught fewer moths.

The 1986 trials had also indicated that the 100:7.5 mixture of Z9-14:Ac and Z,E9,12-14:Ac may be more attractive than the 100:5 mixture. In order to check this and provide a further comparison between the five-component blend and the two-component blend a trial was undertaken in which 5, 7.5, or 10% Z,E9,12-14:Ac was added to the two- and five-component mixtures (Table 8). In each case the five-component mixture caught significantly more moths, confirming previous results. There was no difference in catch between those with mixtures containing 5 or 7.5% Z,E9,12-14:Ac, although the addition of 10% reduced catch significantly in both cases.

The only newly identified compound to show any potential for increasing attractancy in the field from the 1986 field tests was Z11-16:Ac. In order to confirm this, the "standard" two-component mixture was compared with mixtures in which between 2.5 and 10% Z11-16:Ac had been added. In each case, the addition of Z11-16:Ac increased catch significantly above that of the two-component mixture, although the actual amount added did not seem to be important (Table 9).

CONCLUSION

This reassessment of the female sex pheromone of the African armyworm, *Spodoptera exempta*, has confirmed the presence of Z9-14:Ac and Z,E9,12-14:Ac, in the ratio of 100:5 (Beever et al., 1975). In addition, four new com-

TABLE 9. CATCHES OF MALE *S. exempta* MOTHS IN TRAPS BAITED WITH STANDARD TWO-COMPONENT MIXTURE COMPARED TO THOSE WITH Z11-16:Ac ADDED (4 REPLICATES, 20 NIGHTS)

Pheromone component (μg)			Mean catch/trap/night ^a
Z9-14:Ac	Z,E9,12-14:Ac	Z11-16:Ac	
1000	75	0	4.2a
1000	75	25	5.9b
1000	75	50	5.7b
1000	75	100	6.4b

^a Actual mean catches/trap/night; means followed by the same letter are not significantly different at the 5% level by DMRT using $\log(x + 1)$ transformed data.

pounds, Z9-14:Ald, Z11-14:Ac, Z9-14:OH, and Z11-16:Ac, were characterized from virgin female ovipositor washings and volatiles. These compounds were present at 1.5, 2, 3.5, and 4% of the major component, respectively. Field tests confirmed that Z9-14:Ac and Z,E9,12-14:Ac are essential for attractiveness and showed that addition of Z11-16:Ac at 2.5-10% of the major component could significantly increase catches. Using a 1 mg loading of pheromone, the other compounds identified had no effect at the naturally occurring level except Z9-14:OH, which significantly reduced catch.

The study was conducted with the intention of maximizing trap catch for use in the East African monitoring system without incurring high cost. For this reason, compounds of commercial purity were used. Z9-14:Ac currently employed in the network contains approximately 2% of the *E* isomer, which could have an effect on behavior. Similarly, it was important that the effective lifetime of the lures was closely related to those used in the regional monitoring system, although the loading used may not have been optimal for testing the effects of the minor components.

Z9-14:Ac, Z11-14:Ac, Z9-14:OH, and Z,E9,12-14:Ac are commonly found in the sex pheromones of Noctuidae and the genus *Spodoptera* in particular (Arn et al., 1986). However, Z9-14:Ald and Z11-16:Ac have only previously been identified in North American species, such as *Spodoptera frugiperda* (Mitchell et al., 1985; Tumlinson et al., 1986), and Z11-16:Ac in *Spodoptera eridania* (Teal et al., 1985) and *Spodoptera sunia* (Bestmann et al., 1988).

The apparent lack of effect of Z9-14:Ald and Z11-14:Ac on trap catch, at the levels tested, does not necessarily mean they do not influence insect behavior. Pheromones are generally considered to be species specific (Cardé and Baker, 1984), so where a number of closely related sympatric species have

pheromone components in common, it may be anticipated that the other, often minor, components could be involved in deterring cross-attraction (Grant et al., 1988; Linn et al., 1987). *Spodoptera triturrata* is sometimes caught in *S. exempta* traps, and so it is conceivable that, in order to ensure species specificity, all the components presently identified, except perhaps Z9-14:OH, will have to be included in the lures. Wind-tunnel studies and further field trials are now in progress in an attempt to elucidate the possible role of the minor components in inter- and intraspecific chemical communication.

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RESPONSE OF MALE POTATO STEM BORER MOTHS,
Hydraecia micacea (ESPER) TO CONSPECIFIC
FEMALES AND SYNTHETIC PHEROMONE
BLENDS IN THE LABORATORY AND FIELD

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Abstract—Behavior of males of *Hydraecia micacea* (Esper) responding to virgin females and to synthetic pheromone blends were investigated in a laboratory wind tunnel. The synthetic blend consisted of saturated 14:Ac (68.9%), Z9-14:Ac (3.4%), E11-14:Ac (14.6%), and Z11-14:Ac (13.1%). Virgin females were significantly better lures than the four-component synthetic blend for most behaviors. By deleting components individually from the four-component blend, Z9-14:Ac, Z11-14:Ac, and saturated 14:Ac were found to be necessary for communication but E11-14:Ac was found to have no effect on typical pheromone-mediated reproductive behaviors. Close-range studies suggested that chemicals of low volatility, released from moths, were important in eliciting copulation attempts. Field studies reinforced laboratory findings regarding the effectiveness of different lures and indicated that *Heliothis* traps were the most effective for monitoring.

Key Words—*Hydraecia micacea*, Lepidoptera, Noctuidae, potato stem borer, sex pheromones, field trapping, reproductive behavior, tetradecenyl acetate, (Z)-9-tetradecenyl acetate, (E)-11-tetradecenyl acetate, (Z)-11-tetradecenyl acetate.

INTRODUCTION

The potato stem borer moth, *Hydraecia micacea* (Esper), is a periodic pest of numerous crops (Deedat et al., 1983). In the northeastern United States and

southern Canada, the major impact of this insect is on corn, which suffers from wilting of the axial leaves due to larvae feeding within the corn stalk. Although eggs are laid on leaves of weed grasses that surround fields, larvae migrate into fields, and consequently the incidence of damage caused by the insect is correlated directly with the presence of suitable grass oviposition sites (Deedat and Ellis, 1983). Population levels are variable between fields, and, while damage is usually restricted to the outer 20 rows, crop loss in some fields can be as high as 80% (Tetor, 1980).

Synthetic sex pheromones of Lepidoptera are used widely as lures for population monitoring and have potential for use in pest control, particularly when the pest species is distributed in isolated, low-density populations (Plimmer, 1982). We felt that the potato stem borer (PSB) was a good candidate for studies aimed at the development of pheromone-based control programs because reports of PSB infestations fit the criteria outlined above. However, prior to the development of control methods, it is necessary that the pheromone-mediated behavior be thoroughly understood. Consequently, we undertook laboratory study on aspects of the reproductive biology that are regulated by semiochemicals for this insect. Here we report the results of studies of the responses of PSB males to females and to various synthetic blends of pheromone components identified in earlier studies (Teal et al., 1983).

METHODS AND MATERIALS

General. Potato stem borers were reared at 25°C as described by West et al. (1985) using a diet modified from Hinks and Byers (1976). The insects were from a laboratory stock into which field-collected males were introduced each year. Insects were sexed as pupae and transferred to separate rooms that had reversed light cycles. The conditions were 14 hr: 10 hr (light-dark) with temperature at 26°C and 24°C (photophase-scotophase) and relative humidity of 55%. Emergence was checked 30 min prior to scotophase, and adults were transferred to nylon screen cages measuring 30 × 30 × 30 cm, which contained cotton wicks soaked with 10% sucrose solution. Insects used for all laboratory trials were 2 to 5 days old.

Chemical Solutions and Lure Formulations. All chemicals used for formulation of synthetic lures were purchased from Sigma Chemical Co. (St. Louis, Missouri) and were purified by high-performance liquid chromatography using a 25 × 2.5-cm (OD) 5- μ m silica column coated in situ with 15% AgNO₃ (Heath and Sonnet, 1980). The compounds were eluted with toluene. All chemicals were at least 99% pure as determined by capillary gas chromatographic (GC) analysis.

Analyses of pheromone component purity were conducted using 30 m ×

0.25 mm ID SPB-1 and Supelcowax 10 columns (Supelco, Bellefonte, Pennsylvania). Ratios of blend components were verified using both columns. Samples were run isothermally in the split mode at 165°C. Hydrogen was used as the carrier gas at a linear flow velocity of 38 cm/sec. A Hewlett-Packard (Avondale, Pennsylvania) 5790 GC equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator was used for all analyses.

White rubber septa (Sigma Chemical Co., St. Louis, Missouri, catalog No. S5509), used as pheromone dispensers, were Soxhlet extracted with methylene chloride for 24 hr and air dried prior to loading. Blends of synthetic compounds (see Table 1) loaded into lures included 500 μg of the four-component blend identified by Teal et al. (1983), which included tetradecenyl acetate (14:Ac) (68.9%), (Z)-9-tetradecenyl acetate (Z9-14:Ac) (3.4%), (E)-11-tetradecenyl acetate (E11-14:Ac) (14.6%), (Z)-11-tetradecenyl acetate (Z11-14:Ac) (13.1%), and blends from which one of the above components had been deleted. Blends were applied in 200 μl of isooctane to the large well of the septum, and septa were allowed to stand in a fume hood for two days to allow for solvent evaporation.

Bioassays. All laboratory studies were conducted 5–8 hr into the scotophase, which coincided with the peak period of pheromone release by females (West et al., 1984). Tests were conducted in a 2.0 \times 1.0 \times 0.5-m Plexiglas flight tunnel housed in a room with the same photoperiod, temperatures, and humidity maintained in rooms used to house adults. The excurrent end of the flight tunnel was fitted to a fume hood controlled by a variable transformer. Air was pulled through the tunnel at 0.5 m/sec and expelled outside the building.

Lures for all studies, including females, and synthetic blends, were housed in an 8 \times 10 \times 10-cm Plexiglas holding cage with aluminum screen on two opposing sides to allow for air flow through the cage. The holding cage for all trials was positioned in the center of the tunnel about 30 cm from the upwind end. All males used were quiescent before the trial. Males were transferred individually to a 2 \times 2 \times 8-cm Plexiglas cage suspended in the center of the tunnel about 15 cm from the downwind end. Males were used once, then discarded, with the exception of males flown to blanks. These insects were flown to a lure that contained pheromone 24 hr after exposure to blank lures. Males were allowed 1 min for activation, a behavior identified by ambulation and wing fanning. If activation did not occur within this time, the moth was discarded. Otherwise, the trial lasted 5 min. Behaviors were recorded on audiocassette tape and later transcribed.

Male response to females was established by using five to six virgin females that had been observed calling for at least 5 min in the holding cage. When the male behavioral repertoire was established, observed behavior frequencies were tabulated in first order, preceding–following transition matrices from which ethograms were derived as outlined by Stevenson and Poole (1976) and Fagen and

Young (1978). To determine if individual transitions occurred greater than chance, $(\text{observed} - \text{expected})/\text{expected}^{0.5}$ must have been greater than chi-square value at $0.05/(dF)^2$ [when the degrees of freedom (dF) equaled 72 as determined from the transition matrix]. The most probable sequence of behaviors was determined using standard normal deviates calculated from significant chi-square values using the binomial test (Siegel, 1956).

When synthetic blends were tested, two rubber septa were enclosed in aluminum screen and suspended on the downwind screen of the cage used to hold lures or females in earlier studies. The role of individual pheromone components was studied by eliminating a single compound from the four-compound blend (Table 1). The percentages of moths exhibiting each behavior to each lure were calculated. Responses to each of the three-component blends, the four-component blend, and to virgin females were compared using a chi-square test of independence with Yates' correction at $P = 0.05$ (Steel and Torrie, 1980).

Field Studies. Six different lures were used in three different traps in a 6×3 factorial design for field studies. The five synthetic lures were the same as those used in flight tunnel studies, and the sixth was a pair of caged virgin females. Synthetic blends were tested using one rubber septum. The trap designs used were Heliiothis Trap (Albany International, Needham Heights, Massachusetts), Pherocon 1-C (Zoecon corp., Palo Alto, California), and a cone-orifice trap (Struble, 1983) that had the rubber septa suspended centrally. Traps were set with all lures standardized at 1 m high.

Trap catches were counted and trap positions were rerandomized every other day from August 8 to 25, 1985, in a field near Alma, Ontario. Traps were positioned in two adjacent fields where the highest PSB infestation occurred

TABLE 1. COMPARISON OF RESPONSES OF MALE POTATO STEM BORERS TO FEMALES AND SYNTHETIC BLENDS IN WIND TUNNEL USING CHI-SQUARE TEST OF INDEPENDENCE WITH YATES' CORRECTION^a

Treatment	N	Percent behavioral response						
		Flight	Orient	Approach	Land	Search	Copulate	Reorient
Females	67	100.0 a	82.1 a	70.1 a	71.6 a	67.2 a	34.3 a	52.2 a
Four-component	41	95.1 a	41.5 b	26.8 bc	17.1 bc	4.8 b	0.0 b	24.4 bc
No 14:Ac	38	86.8 b	42.1 b	13.2 c	0.0 d	0.0 c	0.0 b	10.5 cd
No Z9-14:ac	37	97.3 a	37.8 b	21.6 bc	5.4 c	0.0 c	0.0 b	23.3 bc
No Ell-14:Ac	36	94.4 ab	55.6 b	36.1 b	25.0 b	13.9 b	0.0 b	41.7 ab
No Z11-14:Ac	40	100.0 a	37.5 b	2.5 d	2.5 c	0.0 c	0.0 b	10.0 d
Blank	41	84.9 b	0.0 c	0.0 d	0.0 d	0.0 c	0.0 b	0.0 d

^aNumbers in the same column followed by the same letter are not significantly different at $P = 0.05$.

along the common hedgerow and extended to a grassy L-shaped strip through one of the fields. Traps were positioned 30 m apart. Septa were not changed, but females were replaced after counts were taken. No traps were filled to capacity when counts were taken. Both lure and trap-catch data were transformed by $(X + 1)^{0.5}$ before being subjected to analysis of variance and Duncan's new multiple-range test (Anonymous, 1982).

Close-Range Behavior. The roles of chemical cues and vision in eliciting copulation attempts were tested using wind-tunnel bioassays. Virgin females were used as the pheromone source to satisfy the assumption that the sex pheromone blend was correct.

Two distances were used to test chemical cues in eliciting copulation attempts. Virgin females were placed in screen tubes, measuring 2 cm in diameter, which were placed inside the holding cage at two distances from the downwind screen of the holding cage. At the farthest position, the females were 2–4 cm from the downwind screen of the holding cage, while they were less than 2 cm from the screen at the closer distance. The holding cage was placed in the flight tunnel as described earlier.

The two conditions to test vision were the presence or absence of a model. The model was a dead male rinsed with acetone, impaled on a fine wire and positioned inside the same holding cage used previously. The wings of the model were spread to simulate the outline of a calling female.

Only responses from males that landed on the holding cage were recorded. Four comparisons were made. Two comparisons tested the effect of the model at each distance, and two tested the effect of distance with and without the model. New models were used with each pheromone source. A test of proportion with the *Z* statistic was used for statistical analysis (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Male Behavior to Virgin Females. The behavioral repertoire of males responding to calling females included activation, random flight, upwind orientation, hovering, landing, searching the site, attempted copulation, casting downwind, reorientation, and stopping (Figure 1). Activation included ambulation, wing fanning, and antennal grooming. Random flight involved movement through the entire volume of the tunnel and included short bouts (< 1 min) of sitting or walking on the tunnel walls. Orientation was identified as wide horizontal and vertical sweeps in the downwind half of the tunnel with slow upwind movement and progressively narrower sweeps closer to the pheromone source. Hovering was indicated by stationary flight within 10 cm downwind of the holding cage, and landing indicated that the male had contacted the holding cage. Searching consisted of a combination of behaviors performed while on

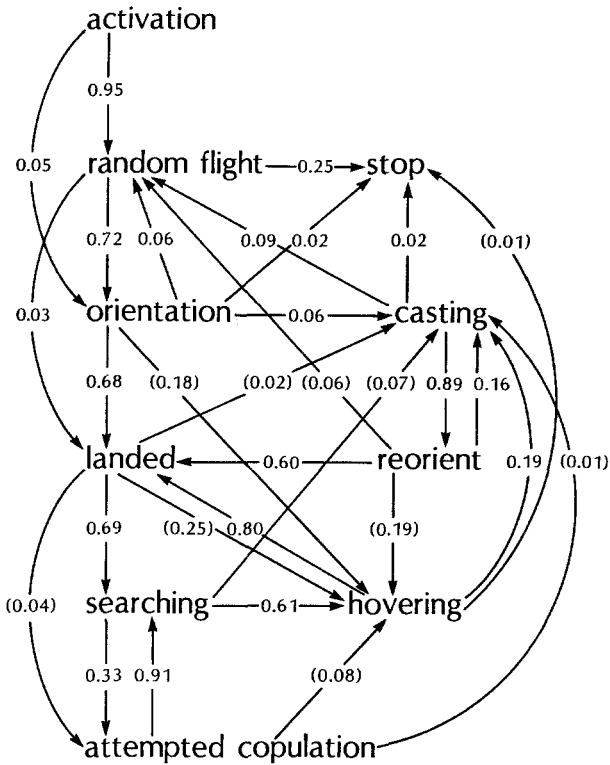


FIG. 1. Ethogram of response of male PSB to calling virgin female PSB in wind-tunnel bioassays. Numbers represent the conditional probability of a moth performing a behavior after exhibiting the previous behavior. Numbers in parentheses represent transitions occurring at a frequency not greater than chance ($N = 67$).

the holding cage that included walking vertically and horizontally and turning both clockwise and counterclockwise. Searching was always accompanied by rapid wing-fanning. Attempted copulation was typically a lateral curving of the abdomen to either left or right. Casting was direct downwind flight, usually in an upward arc, after the male had oriented to the lure. Reorientation was upwind flight to the pheromone source after having oriented previously. Males were regarded as stopped when they sat on a wall for at least 1 min.

Behavioral responses of males to calling females were highly variable (Figure 1), but use of the binomial test allowed for the identification of the most probable sequence of behaviors (Figure 2). PSB males typically oriented to the females within ca. 1 min, with landing on the source usually initiating a cyclic series of behaviors. Landing led to searching, which was interspersed with

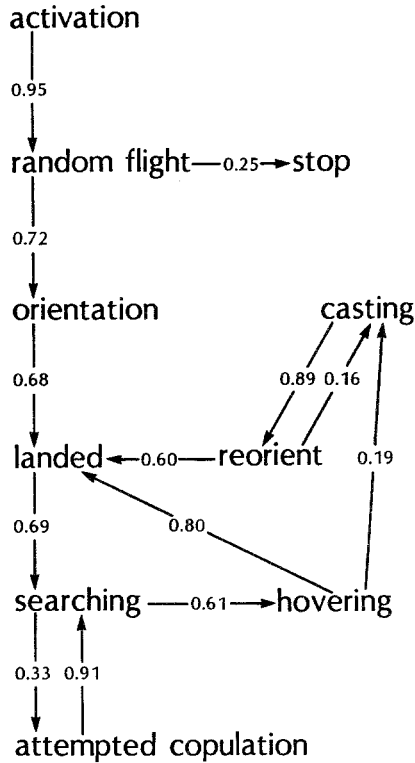


FIG. 2. Ethogram of the most probable sequence of response of male PSB in the wind tunnel. Numbers represent the most probable sequence of behaviors from the comprehensive ethogram of Figure 1 ($N = 67$).

attempted copulations. Copulation attempts were made only when the female was opposite the male on the downwind screen of the holding cage. Subsequent hovering was followed by a return to landing. This cycling could last the duration of the trial. Some males had a larger cycle of behaviors that included casting after hovering and then reorienting back to the source. Male PSB were usually active through the end of the trial. Blank lures elicited only random flight in the wind tunnel, indicating that upwind orientation behavior was induced by pheromones.

Response to Synthetic Blends in the Wind Tunnel. No blend of synthetic components was as effective as females in eliciting behaviors (i.e., orientation, landing, copulation) necessary for successful mating in a single attempt (Table 1). This reduction in response was not due solely to the significant decreases in the number of males orienting upwind but due also to combined reductions in

the numbers of males orienting, landing, and attempting to copulate. Studies with other noctuid species, for example, *Heliothis phloxiphaga* Grote and Robinson (Raina et al., 1986) and *H. virescens* (Teal et al., 1986), have shown that synthetic blends of pheromone components are capable of reproducing or surpassing virgin females in attractiveness and ability to elicit reproductive behaviors. Consequently, the four-component pheromone blend used in this study and identified by Teal et al. (1983) does not accurately represent the complete sex pheromone released by as many as five to six females of the potato stem borer.

Differences in behavioral response between the synthetic lures and calling females could be due to components missing from the blend and/or inaccurate component ratios and release rates. Reevaluations of sex pheromone blends of other species have often resulted in the addition of components which, although present in small quantities, were important for either maximizing pheromone-induced behaviors in the wind tunnel or for increasing trap catch (Bjostad et al., 1984; Sparks et al., 1979).

Subsequent studies by Teal and Tumlinson (1987) indicated the presence of picogram amounts of a series of 14-carbon alcohols in ethyl ether extracts of the pheromone gland of calling female PSB. These alcohols are the immediate precursors of the acetates identified for the PSB by Teal et al. (1983) and may be released in the pheromone blend by female PSB in minute amounts. Consequently, these compounds could be critical for effective signaling, while still being precursors for the acetate compounds. This supposition is supported by a study of *H. phloxiphaga* conducted by Raina et al. (1986) in which minute amounts of (*Z*)-11-hexadecen-1-ol, a compound known to be the immediate precursor of the aldehyde present in the greatest amount in the volatile pheromone blends of *Heliothis* spp. (Teal and Tumlinson, 1987), is critical for effective communication. Other unidentified components also may be present in the pheromone blend of PSB females.

Ratios of components have been reported to differ between gland extracts and volatile blends in other Noctuidae (Bjostad et al., 1980; Teal et al., 1985, 1986) and in other lepidopteran families (Hill et al., 1975; Roelofs et al., 1975; Hill and Roelofs, 1981). Evaluation of the volatile pheromone blend released by calling PSB females will allow for the elucidation of the actual components, ratios, and release rates of the pheromone.

Although none of the synthetic blends tested was as effective as females in inducing males to perform behaviors associated with pheromone communication (i.e., orientation, landing, etc.), most blends were as effective as females in causing males to enter flight (Table 1). No behaviors were affected when E11-14:Ac was deleted from the four-component blend. This suggests that E11-14:Ac does not play a role in chemical communication by this species. However, we cannot exclude the possibility that this compound affects as yet

undefined behaviors of conspecific males or that it may play a role in reproductive isolation between the PSB and other closely related species. Fewer males approached to within 10 cm of lures that did not contain Z11-14:Ac than when either the four- or three-component blend that lacked E11-14:Ac was tested. The decrease in the number of approaches also resulted in fewer males landing when Z11-14:Ac was deleted with respect to the blend lacking E11-14:Ac. Consequently, Z11-14:Ac appears to affect close range orientation by males. Deletion of 14:Ac resulted in significant reductions, with respect to either the four-component or three-component blend lacking E11-14:Ac, in the number of males that landed on the holding cage. Similarly, the blend lacking Z9-14:Ac was less effective than the blend lacking E11-14:Ac in inducing males to land. However, no single compound appeared to be responsible for the release of a specific behavior and, consequently, the totality of the blend may be as important as the blend constituents in stimulating pheromone-mediated reproductive behavior.

Field Trapping Studies. Results of field trapping studies that employed different lures and trap types are given in Table 2. Trap design accounted for the greatest variation in catch as is indicated by the fact that *Heliothis* traps always caught some males when baited with any lure, whereas males were captured in double-cone traps only when these traps were baited with females. The success of the *Heliothis* trap was probably the result of the large lower aperture. After approaching or landing on lures in flight-tunnel studies, males commonly cast back downwind. Therefore, moths that flew up and away from the lures positioned beneath these traps would fly into the trap. Once inside, the lower rim probably prevented most moths from crawling down and out of the trap. The Pherocon trap requires insects to land in order to be captured. Consequently, lures that did not induce landing or close-range approach in the flight

TABLE 2. MEAN NUMBER OF MALE PSB CAPTURED PER SAMPLING PERIOD IN DIFFERENT TRAP TYPES BAITED WITH VARIOUS PHEROMONE LURES

Lure	Trap type ^a		
	Heliothis	Pherocon 1-C	Double cone
Female	47.0 a	3.4 c	1.8 d
Four-component	36.7 a	2.6 c	0.0 d
Minus E11-14:Ac	44.6 a	2.6 c	0.0 d
Minus Z9-14:Ac	10.4 b	0.0 d	0.0 d
Minus 14:Ac	2.0 c	0.0 d	0.0 d
Minus Z11-14:Ac	3.0 c	0.0 d	0.0 d

^aValues followed by the same letter are not significantly different by Duncan's multiple-range test.

tunnel (i.e., those that lacked either Z11-14:Ac or 14:Ac) were ineffective in these traps. Pherocon traps baited with lures lacking Z9-14:Ac also did not capture males in field studies. This may be explained by the low incidence of landing in the flight-tunnel study, coupled with the chance of escape from the trap glue by these large moths.

It was expected that cone-orifice traps would catch moths only when baited with females for two reasons: first because males would spend considerable time searching and crawling into these traps, and second because the levels of activity to the synthetic blends were significantly lower for all behavioral criteria examined in the flight tunnel than those recorded when females were tested. The relatively low number of insects caught in cone-orifice traps when females were used as lures may be explained by plume shape. *Heliothis* traps and Pherocon 1-C traps probably had the least plume disruption because their exposed lures probably produced an omnidirectional effect with respect to wind. Cone-orifice traps were unidirectional with respect to wind, however, and probably had poor flow through the trap most of the time because of variable wind direction. Struble (1983) found cone-orifice type traps generally had lower catches of Lepidoptera than omnidirectional designs.

There was also a good correlation between the capture of males in *Heliothis* traps with different lures and the ability of those lures to elicit reproductive behaviors from males in wind-tunnel bioassays. Numbers of males caught using the four-component blend or the blend missing E11-14:Ac were not significantly different. The fact that neither of these synthetic lures was different from females suggests an inconsistency between these field data and wind-tunnel studies. However, wind-tunnel studies employed groups of five to six females caged in a relatively large container that enabled females to call undisturbed, while field studies employed only two females housed in small cages, which increased contact between insects. Consequently, the difference between the laboratory and field may be the result of much smaller amounts of pheromone released by females used in field studies. Deletion of Z11-14:Ac from lures used in the field caused a reduction in captures that is correlated with the fact that this compound affected both the approach and landing of insects in flight-tunnel studies. Similarly, deletion of 14:Ac resulted in reduced capture. The intermediate numbers captured in *Heliothis* Traps baited with lures that did not contain Z9-14:Ac reflect the fact that while the number of males that both approached and landed on these lures in the flight tunnel was reduced with respect to more effective blends, the reduction was not significant.

Close-Range Reproductive Behavior. The fact that few insects landed or performed subsequent searching and that none attempted to copulate in response to any of the synthetic blends in the wind tunnel, coupled with the results of field studies that indicated the four-component blend and three-component blend lacking E11-14:Ac were as effective as females in traps, suggested that factors

other than the compounds identified from the gland extracts played a role in the reproductive behavior of these insects. Therefore, we conducted studies on the close-range reproductive behaviors of these moths. There were few attempted copulations when caged females were farther than 2 cm from the downwind screen of the holding cage and no behavioral differences between presence or absence of the model at that distance (Figure 3). Copulation attempts increased when the female was less than 2 cm from the downwind screen, but the increase was significant only when the model was absent (Figure 3).

Clearly, factors associated with proximity of the sexes were important in eliciting copulation attempts. These factors were probably chemical, since tactile cues were eliminated by the two layers of aluminum screen that separated the sexes. Since three pairs of males were observed attempting male-male copulations in preliminary trials when females were positioned greater than 2 cm from the downwind holding cage screen, these chemical cues do not appear to be restricted to the female sex pheromone blend. Chemicals of low volatility released from moth scales may be a cue. Acetone-washed males used as models may have been stripped of these chemical cues.

Grant et al. (1987) reported contact pheromones found in scale extracts of white-marked tussock moth females, *Orgyia leucostigma* (J.E. Smith), that released copulatory behavior from the males. Similarly, Ono (1981) found the presence of scales could induce interspecific copulation attempts in three species

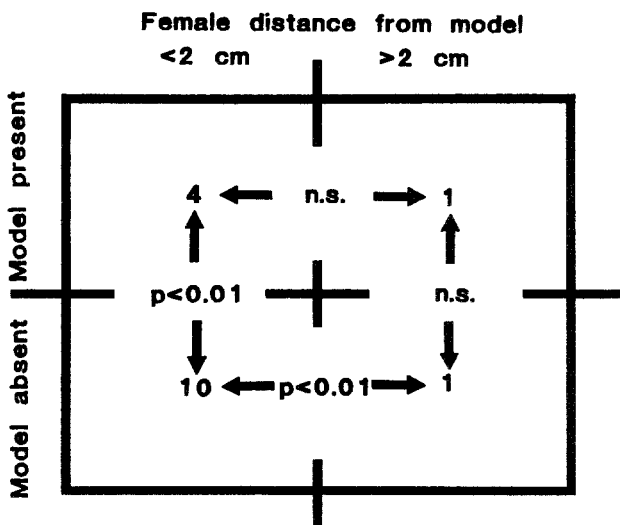


FIG. 3. Number of male PSB attempting to copulate with caged female PSB both with and without the model, and at two distances, in the wind tunnel. Arrows indicate pairwise comparisons made using the Z statistic at $P = 0.05$ ($N = 10$ in each cell).

of phycitid moths. Male PSB may require similar compounds to release the copulatory response. If so, present studies indicated that for the PSB, chemicals can be perceived at short distances and that male contact with the female is not necessary to elicit a copulatory response.

Visual stimuli played a secondary role to chemical cues in eliciting copulation attempts because any factor that decreased copulation attempts with the model present was more important than vision. It was expected that the model with females less than 2 cm distant would elicit the same behaviors as males responding to unrestricted females. However, the reverse occurred. Inhibition in response to the model at close range may be the result of either chemicals from the model that inhibited behavior, or because close-range chemicals from a female less than 2 cm away were not perceived due to disrupted air flow around the model. It is not possible to determine from these data, however, what role visual cues do play in eliciting copulation attempts from male PSB. The role of visual cues is variable in the Noctuidae, but for most, it is secondary to an accurate sex pheromone blend in eliciting close-range behaviors (Shorey, 1964; Shorey and Gaston, 1970; Carpenter and Sparks, 1982).

In conclusion, our studies suggest that the four compounds identified from extracts of the pheromone gland of females of *H. micacea* are of importance in semiochemical communication. However, disparities observed in flight-tunnel studies between synthetic lures and females suggest that this blend does not accurately represent the one released by females. This could be the result of the lack of as yet unidentified compounds important for communication, inappropriate release rate, or incorrect ratios, or all of these factors.

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CRUDE EXTRACTS OF ASTERACEOUS WEEDS Growth Inhibitors for Variegated Cutworm

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Abstract—Petrol and ethanolic extracts of six asteraceous weeds were added to artificial diets to screen for growth inhibition and mortality of the variegated cutworm, *Peridroma saucia* (Hbn). Petrol and ethanolic extracts of *Artemisia tridentata* and *Chamomilla suaveolens* and ethanolic extracts of *Chrysothamnus nauseosus* and *Centaurea diffusa* severely inhibited larval growth at five times the natural concentrations. The two *C. suaveolens* extracts and the ethanol extract of *A. tridentata* were active at the natural concentration (100%) and were further examined at 20, 40, 60, and 80% of this level. Inhibition of larval growth was directly related to concentration for each of the three extracts tested. EC₅₀s (effective concentration to inhibit growth by 50% relative to controls) for the three extracts were 36–42% of the naturally occurring level in the plants. Nutritional indices were calculated for second-instar *P. saucia* feeding on the active *A. tridentata* EtOH extract and the petrol extract from *C. suaveolens*. Addition of the active *A. tridentata* EtOH or the *C. suaveolens* petrol extract to the diet resulted in significant reduction in the relative growth rate of larvae, although the *A. tridentata* extract was much more inhibitory. Dietary utilization was significantly lower for larvae fed the *A. tridentata* EtOH extract.

Key Words—*Artemisia tridentata*, *Chamomilla suaveolens*, *Chrysothamnus nauseosus*, *Peridroma saucia*, Lepidoptera, Noctuidae, growth inhibition, plant extracts, pest control.

INTRODUCTION

Insect growth inhibitors (e.g., feeding deterrents) may provide effective tools for integrated crop management systems by protecting crops from herbivory

while avoiding destruction of the beneficial insect complex (Bernays, 1983). For example, recent studies suggest that some of the pyrethroids show promise at sublethal doses because they deter feeding and inhibit insect growth (Kumar and Chapman, 1984; Dobrin and Hammond, 1985).

Plants provide an abundant source of new chemicals to examine as pest control agents, including growth inhibitors. Recently, the presence of novel pesticidal compounds from some plants has been examined (Delle Monache et al., 1984; Villani and Gould, 1985; Lane et al., 1987). The acute insecticidal activity of many plants is known (Crosby, 1971), but only more recently have they been examined for insect growth inhibitors (Grainge et al., 1985).

The highly evolved plant family Asteraceae includes many temperate weeds and is a rich source of chemical constituents (Heywood et al., 1977). For example, asteraceous plants contain sesquiterpene lactones that inhibit larval growth in several insect species (Mabry and Gill, 1979; Isman and Rodriguez, 1983).

Chronic feeding bioassays are used to assess the insect growth-inhibitory activity and lethality of ethanolic and petrol extracts from big basin sagebrush (*Artemisia tridentata* Nutt.), diffuse knapweed (*Centaurea diffusa* Lam.), pineappleweed [*Chamomilla suaveolens* (Pursh) Rydb. = *Matricaria matricarioides* (Less.) Porter], common rabbitbush [*Chrysothamnus nauseosus* (Pallas) Britton], tansy ragwort (*Senecio jacobaeae* L.), and goat's-beard (*Tragopogon dubius* Scopoli). (Plant nomenclature follows Taylor and MacBride, 1977.) The target insect for these bioassays is a polyphagous noctuid, the variegated cutworm, *Peridroma saucia* (Hbn). *P. saucia* is an important economic pest, occasionally causing serious damage to horticultural crops in Canada (Beirne, 1971) and the United States (Capinera, 1978; Crumb, 1929). The overall objective of this research is to screen plant extracts as potential pest control agents.

METHODS AND MATERIALS

Plant Extracts. Air-dried plants, collected from southern British Columbia, were finely ground in a Wiley mill. Plant species, parts extracted, location, and the harvest dates are listed (Table 1). Powdered plant material (200 g) was extracted for 24 hr in 1 liter of either petrol (boiling range 30–60°C) or 95% aq EtOH at room temperature (21°C). The slurry was filtered, rinsed, and the extracts reduced under vacuum to 10–60 ml, depending on their viscosity.

Initial Screening. Extracts fivefold those naturally occurring (dry weight plant powder/dry weight artificial diet) were admixed with the dry portion of the artificial diet (Bioserv Inc., Frenchtown, New Jersey; No. 9682), and the carrier solvent was removed in a fume hood. Controls consisted of artificial diet similarly treated with the carrier solvent alone. Upon hatching, two neonate *P. saucia* larvae from our laboratory colony were placed on ca. 2-g (wet weight)

TABLE 1. PLANT SPECIES, COMPONENTS EXTRACTED, HARVEST LOCATIONS IN BRITISH COLUMBIA AND DATES OF MATERIAL BIOASSAYED IN INITIAL SCREENING^a

Plant species	Components extracted	Location	Harvest date
<i>Artemisia tridentata</i>	stms, lvs, fls ^a	Summerland	10-83
<i>Centaurea diffusa</i>	stms, lvs, fls	Kamloops	10-83
<i>Chrysothamnus nauseosus</i>	stms, lvs	Keromeos	5-84
<i>Chamomilla suaveolens</i>	whole plant	Vancouver	5-84
<i>Senecio jacobaea</i>	stms, lvs	Abbotsford	5-84
<i>Tragopogon dubius</i>	stms, lvs, fls	Hedley	5-84

^a stms = stems, lvs = leaves, fls = flowers. *A. tridentata* and *C. nauseosus* consisted of 1-year-old growth. All other species consisted of the entire aboveground portion of the plant at the time of harvest (the roots were included only with *C. suaveolens*).

aliquots of diet in 30 ml plastic cups. Fifteen cups per treatment and 15 treatments were randomized in clear plastic boxes at room temperature. For the tabulated presentation, larval growth was measured as a percentage of the controls after 14 days, based on live larval weights. For the analysis, live larval weights were \log_{10} transformed prior to an analysis of variance and mean larval weights were separated using Tukey's studentized range (HSD) test.

Plant Powder and Residue Experiment. The plant residue remaining from the initial extraction and the unextracted plant powders were assayed for bioactivity on the *P. saucia* neonates. Biological activity observed in the residue, but not present in the initial extract screening, could then be further examined. The dry portion of the diet consisted of 50% Bioserv No. 9682 with an equivalent portion of residue or plant powder (1:1 w/w). Control diet was prepared using one part powdered cellulose (alphacel) to one part artificial diet. An additional treatment consisted of the standard diet (full portion) without cellulose. This treatment was used to determine the effect of the control diet, diluted with cellulose, on larval growth. The experimental design was the same as in the previous experiment.

Second Screening. The most inhibitory extracts were chosen for a further feeding bioassay. Artificial diets were freshly prepared using natural concentrations (dry wt plant/dry wt diet) of the plant extracts. Control diets were treated with petrol alone. *P. saucia* neonates were individually placed on ca. 1 g of diet ($N = 25$) and allowed to feed for 11 days and then weighed. Surviving larvae were placed on control diet to determine the persistence of growth inhibitory effects through pupation to adult emergence.

Dose-Response Experiment. Four concentrations (20, 40, 60, and 80% of natural conc. dry wt/dry wt) of the ethanolic extract of *A. tridentata* and both

extracts of *C. suaveolens* were assayed as above with ethanol and petrol controls. *P. saucia* larvae were counted, weighed after 15 days, and then allowed to feed on the control diet until pupation. The pupation and emergence were recorded for each treatment.

Sensitivity of Older P. saucia Larvae. To determine how the biological activity of the plant extracts was influenced by larval age, another dose-response experiment was initiated with older caterpillars. Neonate *P. saucia* larvae were fed for six days on the standard control diet. The resulting second-instar larvae (ca. 7 mg) were then transferred to the treatment diets ($N = 25$). The bioassay and analysis were as previously described in the neonate *P. saucia* dose-response experiment.

Detailed Growth Analysis of P. saucia Larvae Feeding on A. tridentata and C. suaveolens Extracts. To distinguish between behavioral and physiological contributions to larval growth inhibition, a detailed growth analysis was initiated on second-instar *P. saucia*. Larvae (10.9 ± 1.5 mg, $N = 15$) were fed diets at their natural concentrations (100% dry wt/dry wt). An EtOH extract of *A. tridentata* and a petrol extract of *C. suaveolens* were compared with the standard diet treated with petrol. The duration of the experiment was 48 hr, although larvae were weighed at 24 hr as well as 48 hr to determine relative feeding and growth rates over the two 24-hr periods. Except where otherwise indicated, all measurements are based on dry weights. Growth indices were calculated as described by Scriber and Slansky (1981).

RESULTS AND DISCUSSION

Table 2 shows the growth inhibitory effects of crude plant extracts at five times the natural concentration (dry wt/dry wt) on *P. saucia* larvae fed artificial diet. Six extracts exhibited sufficient antibiosis (*sensu* Painter, 1951) to advance to the second screening. No larvae survived on the diets with ethanolic and petrol extracts of *A. tridentata* and *C. suaveolens* and the ethanolic extracts from *C. nauseosus* and *C. diffusa* were similarly active. The *S. jacobaeae* ethanol extract also was significantly different from the control, but it was not considered sufficiently active for further screening.

The results of the unextracted plant powder and residue experiment are shown in Table 3. The efficiency of the extraction process in removing inhibitory factors is shown by testing the extracted plant material for remaining larval growth inhibition. When diet with unextracted plant powder severely inhibited *P. saucia* larval growth, at least one of the extracted residues was shown to have had the growth inhibitory agents removed. Specifically, the diet containing the unextracted *C. suaveolens* plant powder produced significantly smaller larval weights and higher mortality than both residues. Larvae fed the extracted residue diets from *C. suaveolens* grew as well or better than control-fed larvae.

TABLE 2. EFFECTS OF WEED EXTRACTS^a INCORPORATED INTO ARTIFICIAL DIET ON GROWTH AND SURVIVAL OF NEONATE *P. saucia* IN AN INITIAL SCREENING BIOASSAY

Plant species	Growth (% of control)		Survivorship (%) ^b	
	Petrol	Ethanol	Petrol	Ethanol
<i>A. tridentata</i>	0c ^c	0c	0	0
<i>C. diffusa</i>	106a	8c	63	3
<i>C. nauseosus</i>	80ab	0c	67	0
<i>C. suaveolens</i>	0c	0c	0	0
<i>S. jacobaea</i>	105a	18bc	90	37
<i>T. dubius</i>	107a	72ab	80	80
Control	100a	100a	77	73

^aExtract concentrations were five times the natural concentration (dry wt/dry wt).

^b*N* = 30.

^cTreatments followed by the same letter are not significantly different, Tukey's studentized range (HSD) test, *P* = 0.05.

In addition, no survivors were observed among the *P. saucia* larvae fed the *A. tridentata* plant powder diet. Moreover, the weights of larvae fed the ethanol-residue diet were not significantly different from the control. The extracts from these residues were also shown to be the most active *P. saucia* larval growth

TABLE 3. GROWTH AND SURVIVAL OF *P. saucia* NEONATE LARVAE FED UNEXTRACTED PLANT POWDER OR RESIDUE^a INCORPORATED IN ARTIFICIAL DIETS

Plant species	Powder		Ethanol residue		Petrol residue	
	Growth ^b	Survival ^c	Growth	Survival	Growth	Survival
<i>A. tridentata</i>	0g ^d	0	121abc	50	19fg	53
<i>C. diffusa</i>	47cdef	87	34defg	77	31efg	70
<i>C. nauseosus</i>	149ab	40	163ab	90	34bcde	50
<i>C. suaveolens</i>	18g	30	114abcd	77	190ab	80
<i>S. jacobaea</i>	15g	30	98abcd	73	145ab	77
<i>T. dubius</i>	96abcd	67	147ab	43	277a	73
Standard diet			272a	70		
Control (with cellulose)			100abcd	80		

^aPlant material incorporated with artificial diet (1:1 dry wt/dry wt).

^bTaken as the percentage of larval growth of the control treatment with cellulose filler simulating the plant material.

^cPercentage of total larval survivors (*N* = 30).

^dTreatments followed by the same letter are not significantly different, Tukey's studentized range (HSD) test; *P* = 0.05.

inhibitors (Table 4). These results demonstrate that when insect growth inhibitors were present in unextracted plant powders, they were nearly always removed by one or both solvents.

P. saucia larvae fed diet without cellulose were, on the average, over two and a half times heavier than those fed diet containing cellulose. Dietary cellulose may reduce growth of *P. saucia* larvae by a dilution of phagostimulatory nutrients or by the physical presence of more fiber in the diet. The addition of plant powders, however, did not always reduce larval growth. Larvae fed seven of the 18 treated diets gained higher weights than the control with cellulose (Table 3). This increase in growth is attributed to the control diet having been diluted 50% with cellulose, which is nutritionally inferior, whereas the extracted powders may contribute nutrients and phagostimulants.

The results of the second screening experiment are comparable to those of the plant powder experiment. Diet containing the ethanolic extract of *A. tridentata* resulted in 100% mortality of *P. saucia* larvae (Table 4). In addition, larvae fed diets of both *C. suaveolens* extracts grew significantly less and had reduced survivorship compared to larvae fed control diet. These results concur with the plant powder experiment in that diets incorporating unextracted *A. tridentata* and *C. suaveolens* powders were the most biologically active towards *P. saucia* larvae.

Larvae surviving the second screening were transferred to control diet to examine the latent effects of growth inhibition. Healthy *P. saucia* moths emerged from all treatments with larval survivors. These treatments, therefore, do not appear to cause any irreversible physiological damage to the growing larvae or pupae. Reproductive viability of the adults has yet to be determined.

TABLE 4. EFFECTS OF SELECTED WEED EXTRACTS INCORPORATED INTO ARTIFICIAL DIET AT NATURAL CONCENTRATIONS ON WEIGHT AND SURVIVAL OF NEONATE *P. saucia* FED FOR 11 DAYS

Plant species	Extract	Larval weight (% of control)	Survivorship (% of total) ^a
<i>A. tridentata</i>	Petrol	63ab ^b	51
<i>A. tridentata</i>	Ethanol	0d	0
<i>C. diffusa</i>	Ethanol	44ab	92
<i>C. nauseosus</i>	Ethanol	31bc	96
<i>C. suaveolens</i>	Petrol	9d	36
<i>C. suaveolens</i>	Ethanol	12cd	8
Control		100a	96

^aN = 25 neonate larvae.

^bTreatments followed by the same letter are not significantly different ($P = 0.05$) using Tukey's studentized range (HSD) test.

Figure 1 shows the results of the dose-response experiment using ethanol and petrol extracts of *C. suaveolens* and the *A. tridentata* ethanolic extract at four concentrations. *P. saucia* larval weight was inversely related to the plant extract concentration for all three crude extracts. The resulting EC_{50} s (effective concentration to inhibit larval growth by 50%) were 36, 39, and 42% for the *C. suaveolens* EtOH and petrol extract and *A. tridentata* EtOH extract, respectively. Larval growth inhibition could result from behavioral factors (e.g., feeding deterrence), physiological factors (e.g., microsomal enzyme suppression), or both.

Survival of *P. saucia* larvae was also concentration dependent for each extract. Survivorship, however, showed a nonlinear response (Figure 1). Pooled results from two dose-response experiments indicate that low dietary concentrations (0–40% natural concentration) of the plant extracts had little effect on survival. The highest dietary concentration (80%) usually resulted in significantly reduced survival. This suggests a threshold concentration for mortality.

Antibiosis increased with concentration, as shown by the results of both survival and growth inhibition (Figure 1). Dose-dependent antibiosis is evident initially as larval growth inhibition and then, at higher doses, as both increased growth inhibition and mortality. The mode of action of the bioactive plant extracts on *P. saucia* larvae transferred to control diet and allowed to pupate and then emerge, showed no apparent physiological effects.

Age-dependent effects were also examined in a dose-response experiment. Six-day-old larvae appear more tolerant to the plant extracts than neonates. For example, even at an extract concentration of 80%, larval mortality was consistently 15% or less; at the same concentration, growth was 33, 52, and 72%

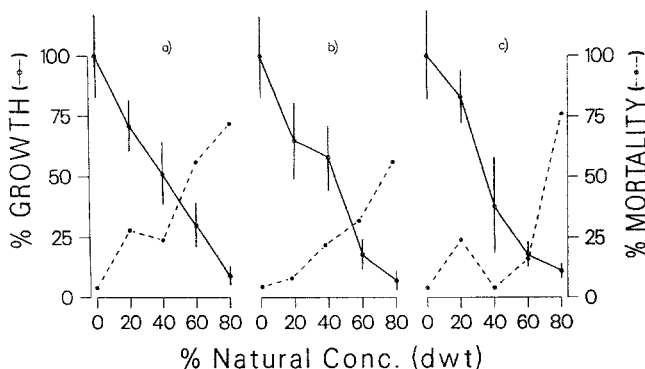


FIG. 1. Percent growth relative to control growth and percent total mortality of *P. saucia* neonates fed ethanolic extracts from (a) *A. tridentata*, (b) *C. suaveolens*, and (c) a petrol extract from *C. suaveolens* admixed to artificial diets ($N = 25$ larvae per concentration with each extract). Standard deviation is given by error bars on the growth points.

of controls for the *A. tridentata* EtOH and the *C. suaveolens* EtOH and petrol extracts, respectively. Decreasing sensitivity to allelochemicals with increasing larval age is well known for other insect species (Reese, 1983; Isman and Duffey, 1982).

Insect growth inhibition could result from behavioral factors (e.g., feeding deterrence), physiological factors (e.g., microsomal enzyme suppression), or both. The results of a detailed growth analysis of second-instar *P. saucia* fed diets containing the *A. tridentata* EtOH extract and the *C. suaveolens* petrol extract are shown in Table 5. The relative growth rate (RGR) is a product of relative consumption rate (RCR) and dietary utilization. The RGR for larvae fed diets with the *C. suaveolens* petrol extract was 70% of the control-diet fed larvae. The majority of this growth inhibition appears to be associated with behavioral factors, as indicated by the significantly lower RCR, whereas dietary utilizations did not differ significantly from the control. The *P. saucia* larvae fed diets with the *A. tridentata* EtOH extracts produced even lower growth rates than larvae fed the *C. suaveolens* extract. This severe growth inhibition, however, appears largely due to physiological effects shown by the extremely low dietary utilization (ECI) even though the RCR was 60% of the controls.

Separate consideration of the RGRs over the 48-hr experiment revealed an interesting phenomenon. The RGR for larvae fed the *C. suaveolens* petrol extracts was 51% of the controls during the first 24-hr period, but in the second

TABLE 5. EFFECTS OF DIETARY *A. tridentata* EXTRACT (EtOH) AND *C. suaveolens* EXTRACT (PETROL) ON SECOND-INSTAR *P. saucia* DIGESTIBILITY OF FOOD (AD), RELATIVE GROWTH RATE (RGR), RELATIVE CONSUMPTION RATE (RCR), AND GROSS (ECI)^a AND NET (ECD)^a DIETARY UTILIZATIONS

Nutritional index	Dietary supplement ^b		
	<i>C. suaveolens</i>	<i>A. tridentata</i>	Control
RGR (mg dry wt/mg dry wt/day)	0.44 ± 0.09 b	0.03 ± 0.15 c	0.58 ± 0.10 a
RCR (mg dry wt/mg dry wt/day)	2.2 ± 0.6ab	1.6 ± 1.2 b	2.7 ± 0.4 a
ECI (mg dry wt/mg dry wt/day × 100)	20.4 ± 6.3 a	-3.6 ± 24.6 b	22.0 ± 5.1 a
ECD (mg dry wt/mg dry wt/day × 100)	44.9 ± 48.2 ab	0.8 ± 59.5 b	52.9 ± 44.6 a
AD (mg dry wt/mg dry wt/day × 100)	59.6 ± 15.2	58.2 ± 33.8	51.8 ± 14.6nsd
N	14	13	15

^aECI = efficiency of conversion of ingested food; ECD = efficiency of conversion of digested food.

^bValues are ± standard deviation. nsd = not significantly different. Means in a row followed by the same letter are not significantly different, Tukey's studentized range (HSD) test, $P = 0.05$.

24-hr period the growth rate accelerated to equal the RGR of the control. In contrast, the RGR for *P. saucia* larvae fed the *A. tridentata* EtOH diet was significantly lower ($P = 0.05$) than the controls for both 24-hr periods at 27 and 29% of control fed larval RGR, respectively. This suggests that growth inhibitors that decrease nutrient utilization in insects may be more persistent crop-protection agents than behavioral feeding deterrents.

Bioactivity of these extracts is not restricted to *P. saucia* larvae. Larvae of another polyphagous noctuid, the alfalfa looper, *Autographa californica* Speyer, tested in a chronic feeding dose-response experiment as described above, resulted in EC_{50} s of ca. 10–20% for all three extracts (unpublished data). These EC_{50} s are about half the observed EC_{50} s for *P. saucia* larvae, and no *A. californica* larvae survived the 60 or 80% concentrations. Unlike *P. saucia* larvae, mortality of *A. californica* appears to be linearly related to concentration when tested at the same levels, even though the relative inhibitory activity of the three extracts was the same for both species. Differences in sensitivity of noctuid species to the same allelochemicals may be related to metabolic and sensory differences. In choice experiments, Jermy et al. (1981) showed that Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say), are inhibited from feeding on leaf disks coated with an ethanolic extract from *A. tridentata*. These results suggest the biological activity of the *A. tridentata* extract may have a broad spectrum of activity on phytophagous insects.

Many secondary metabolites have been isolated from *A. tridentata* (Table 6), and some are reported to have insect growth and feeding inhibitory activity. Monoterpenes (Buttkus et al., 1977; Welch and McArthur, 1981), coumarins (Brown et al., 1975; Murray et al., 1982), and sesquiterpene lactones (Kelsey and Shafizadeh, 1979) have been previously extracted as major components of *A. tridentata*. Jermy et al. (1981) bioassayed the sesquiterpene lactone deacetylmatricarin and reported good feeding deterrent activity against larval Colorado potato beetle. They noted that significant feeding deterrent activity remained in the extract even after removal of deacetylmatricarin, indicating that more than one compound was responsible for the deterrence. Wisdom et al. (1983) tested five sesquiterpene lactones against *H. zea* and found that only a guaianolide from *A. tridentata*, dehydroleucodin, significantly reduced growth. Table 6 lists some of the candidate active ingredients that may also have a role in the biological activity of the *A. tridentata* extract.

Screening crude plant extracts in the laboratory is the initial step in their development as pest control agents. Potent insect growth inhibitors may have a use where resistance to insecticides is a problem (Bernays, 1983) and where pest managers are integrating biological and cultural controls into crop protection strategies. Further investigations of the *A. tridentata* extract will be focused on field studies to assess the practical application of these extracts.

TABLE 6. PHYTOCHEMICAL CONSTITUENTS PREVIOUSLY ISOLATED FROM *Artemisia tridentata*

Monoterpenes ^a	Sesquiterpene lactones ^b	Coumarins ^c
Camphor	Matricarin	Esculin
1,8-Cineole	Tatridin A, B, C	Umbelliferone
Delta-3-carene	Deacetyxmatricarin	Cichoriin
Santoliny ester	Deacetylmatricarin	Isoscopoletin
Alpha-pinene	Ridentin	Scopoletin
Camphene	Dentatin A, B	Scoparon
	Dehydroleucodin	Esculetin
	Arbusculin A, B, C	Artelin
Flavonoids ^d		
	Quercetagenin 3,6-dimethyl ether	
	Quercetagenin 3,6,7-trimethyl ether	
	Kaempferol 3,6,7-trimethyl ether	
	Luteolin	
	Luteolin-7-O-glucoside	
	6-Methoxy luteolin	
	Axillarin	

^aButtkus et al. (1977) The monoterpenes listed comprise 80% of the essential oils.

^bSeaman (1982).

^cBrown et al. (1975) and Murray et al. (1982) These compounds are 80% of the phenolic fraction of an *A. t. spp. vaseyana* extract.

^dRodriguez et al. (1972).

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IDENTIFICATION OF LEEK-MOTH AND
DIAMONDBACK-MOTH FRASS VOLATILES
THAT STIMULATE PARASITOID,
Diadromus pulchellus

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Abstract—*Acrolepiopsis assectella* and *Plutella xylostella* frass volatiles, trapped on Tenax GC, were examined by capillary gas chromatography. In both moths, the same three disulfides, dimethyl, dipropyl, and methyl propyl, were the most abundant substances, but in different proportions. The synthetic disulfides elicited the same behavioral response by the parasitoid, *Diadromus pulchellus* as frass. The plant origin of these substances is discussed.

Key Words—*Diadromus pulchellus*, Hymenoptera, Ichneumonidae, disulfides, frass, *Acrolepiopsis assectella*, *Plutella xylostella*, Lepidoptera, Acrolepiidae, Plutellidae, leek, *Allium porrum*, cabbage, *Brassica oleracea*.

INTRODUCTION

The odor of the leek, *Allium porrum* L., is characterized by the presence of specific sulfur-containing substances, thiosulfonates, that rapidly degrade into disulfides. Similarly, crucifers emit isothiocyanates that are specific sulfur-containing substances.

The locomotor activity of *Diadromus pulchellus* Wesm. (Hymenoptera: Ichneumonidae), an endoparasitoid of the young nymph of the leek moth, *Acrolepiopsis assectella* Zell. and of the diamondback moth, *Plutella xylostella* L.

(Thibout, 1988) is stimulated by the odor of both leek and cabbage, especially by that of leeks when they are attacked by leek moth larvae. Paradoxically, among the substances present in these odors, the least specific are the disulfides, which are the most active in olfactometry and electroantennography (Lecomte and Thibout, 1983, 1984, 1986; Lecomte and Pouzat, 1985). In fact, the frass odor from host larvae is more stimulatory for *D. pulchellus* than the odor from the plants (Lecomte and Thibout, 1986; Thibout et al., 1988). Frass odor has been analyzed by gas chromatography, and a simple test enables a determination of the stimulation by the volatile substances.

METHODS AND MATERIALS

Instrumentation. Analytical gas chromatography (GC) was performed with a Varian 3300 chromatograph equipped with on-column injection, FID and FPD detectors in the sulfur mode, and a S.G.E. fused silica WCOT column, 25 m long, 0.32 mm ID, 0.5 μm thick film of BP 20, equivalent to Carbowax 20 M.

Materials. Dipropyl disulfide (DS P₂) and dimethyl disulfide (DS M₂) were obtained from Merck. Allyl isothiocyanate (NCS Al) and dimethyl trisulfide (TS M₂) were purchased from Kodak.

Methyl propyl disulfide (DS MP) was synthesized by oxidizing an equimolar mixture of the CH₃S⁻ and C₃H₇S⁻ ions through controlled potential electrolysis carried on a gold electrode of great area in aprotic dipolar media (*N,N*-dimethyl acetamide). These thiolate ions had been previously generated in the same solvent from symmetrical disulfides by electrochemical reduction (Iversen and Lund, 1974). Reoxidation yielded a mixture containing about 30% dimethyl disulfide, 40% methyl propyl disulfide, and 30% dipropyl disulfide. The absence of other substances was verified by NMR spectroscopy and GC.

Trapping and Isolation. Head-space frass volatiles emitted at ambient temperature in a 0.005-m³ closed receptacle were trapped for 1 hr on a cartridge (4 mm diameter, 20 mm long) containing Tenax GC 60–80 mesh (30 mg) directly connected to a Gillian LFS 113 pump. Trapped substances were extracted with ether (1 ml) and then injected or submitted to the bioassay.

Identification. Sulfur compounds were identified by their GC retention times in comparison to those of authentic samples. Most of these reference substances had previously been identified in the degraded odor of *Allium* (Auger et al., 1989).

Bioassay. The action of odors on the host searching behavior of *D. pulchellus* was evaluated by measuring its locomotor activity directly in the rearing screened cage (40 × 30 × 24 cm) containing approximately 400 adults of both sexes. Every 30 sec for 20 min, the numbers of parasitoids on two 1-cm² pieces of paper were noted; one piece was impregnated with the test substance, the

other with the solvent (control). The results of 40 observations were compared by the Wilcoxon test.

The source of *D. pulchellus* and breeding and laboratory conditions have been described in detail elsewhere (Lecomte and Thibout, 1983, 1984, 1986): 16:8 hr light-dark, 25°C light and 15°C dark, relative humidity 60 ± 5% light and 70 ± 5% dark.

Frass was removed from the breeding cages of each species. Their larvae were fed on their respective host plants: cabbage, *Brassica oleracea* L., for *P. xylostella* and leek, *A. porrum*, for *A. assectella* under conditions 18:6 hr light-dark, 26°C light and 18°C dark, relative humidity 60 ± 5% light and 75 ± 5% dark. Frass was refrigerated in a closed receptacle until the required amount (1 g) was obtained.

RESULTS

The odor of *A. assectella* frass includes essentially sulfur compounds (Figure 1), of which the three most abundant are dimethyl, dipropyl, and methyl propyl disulfides. The propyl radical was more abundant than the methyl radical, with the propyl-methyl ratio varied from 3 to 10 between experiments.

The odor of *P. xylostella* larval frass contained the same three sulfur compounds, but the methyl group was about as abundant as the propyl group, and some dimethyl trisulfide was also present (Figure 2).

Dimethyl and dipropyl disulfides as well as the odors of the *A. assectella* and *P. xylostella* frass tested on the activity of *D. pulchellus* all had a significant effect, since the number of parasitoids observed on the papers with odor was always greater than that observed on the control papers ($P < 0.001$) (Table 1).

DISCUSSION

Dimethyl, dipropyl, and methyl propyl disulfides from both *A. porrum* and *B. oleracea* seem to be responsible for the effect of *A. assectella* and *P. xylostella* frass on the activity of *D. pulchellus*. Humid frass emits greater quantities than dry frass, as has been reported earlier (Van Leerdam et al., 1985).

The variation of the propyl-methyl ratio in *A. assectella* frass could not be related to frass age or water content. This variation is perhaps due to the part of the leek consumed by the larvae, since the proportions of sulfur volatiles of *Allium* differ, depending on the part of the plant (Mackenzie and Ferns, 1977). The precursors, methyl cysteine sulfoxide, and propyl cysteine sulfoxide, yield thiosulfonates by an enzymatic reaction when the leaves are crushed. These compounds degrade rapidly, especially into disulfides (Auger et al., 1989). In addition, microorganisms can also degrade these precursors (Murakami, 1960).

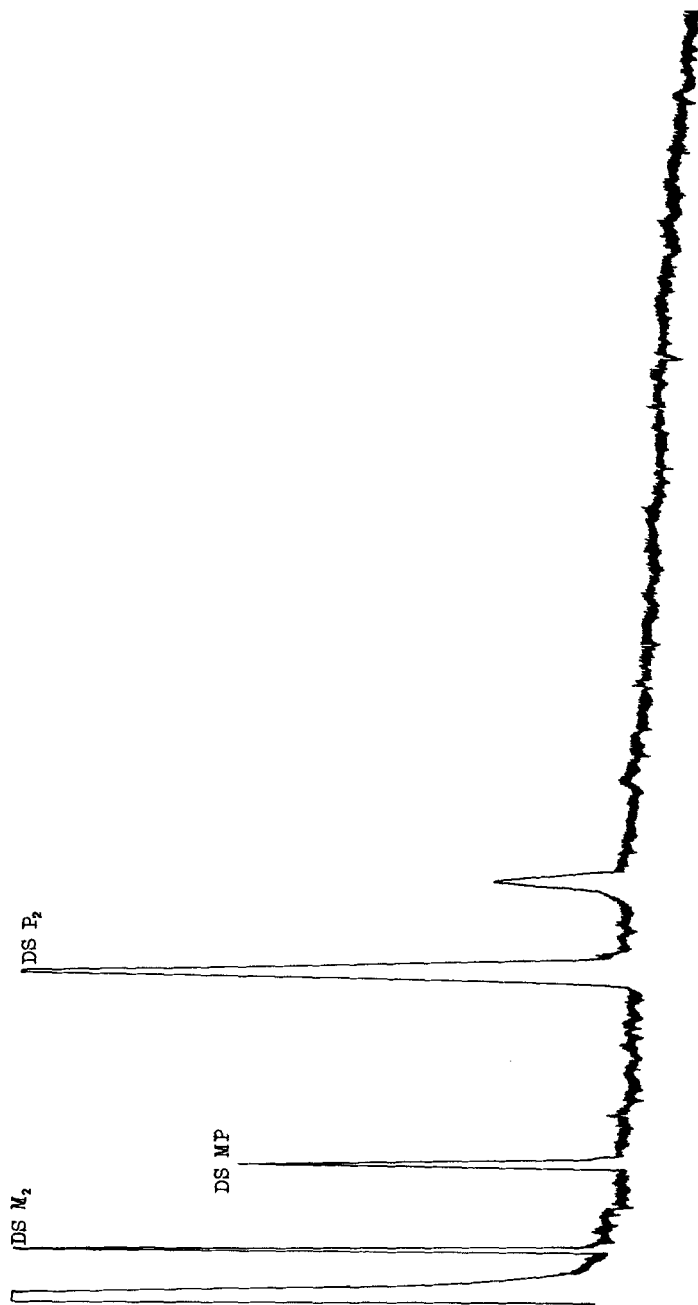


FIG. 1. Gas chromatogram of the leek moth frass volatiles trapped on Tenax. Column: 25 m \times 0.33 mm ID; temperature program: 1 $^{\circ}$ C/min from 80 $^{\circ}$ C to 150 $^{\circ}$ C; on-column injection; FP detector. DS M₂ = dimethyl disulfide; DS MP = methyl propyl disulfide; DS P₂ = dipropyl disulfide.

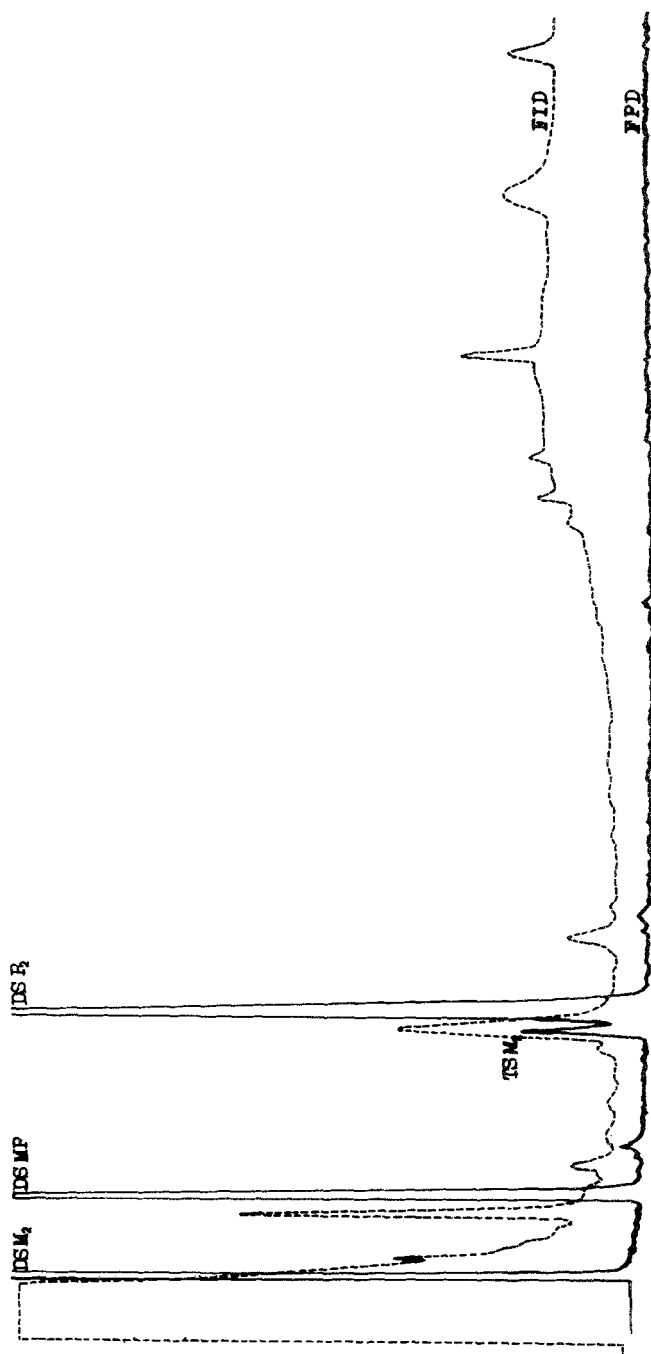


FIG. 2. Gas chromatogram of the diamondback moth frass volatiles trapped on Tenax. Column 25 m \times 0.33 mm ID; temperature program: 1 $^{\circ}$ C/min from 80 $^{\circ}$ C to 150 $^{\circ}$ C; on-column injection; FP and FI detectors. DS M₁ = dimethyl disulfide; DS MP = methyl propyl disulfide; DS P₂ = dipropyl disulfide; TSM₁ = dimethyl trisulfide.

TABLE 1. EFFECT OF HOST FRASS VOLATILES AND DISULFIDES ON *Diadromus pulchellus* ACTIVITY

	Frass volatiles		Synthetic disulfides	
	<i>A. assectella</i>	<i>P. xylostella</i>	DS M ₂	DS P ₂
R ^a	4.5	11.0	3.2	7.0

^aR = ratio of the numbers of parasitoids on two papers, to one of which volatiles were applied, the other served as a control.

Therefore, it is not surprising that the sulfur compounds of *Allium* are transformed to disulfides during larval digestion. The propyl group is predominant in the leek (Auger et al., 1989), much more so than in the frass of the larvae that consume the same leek.

The result obtained for *P. xylostella* frass is very similar to the above, even though the odor of crucifers includes not only disulfides, but much more specific sulfur compounds, isothiocyanates (Bailey et al., 1961). For example, the odor of the cabbage *B. oleracea*, cut and analyzed with the techniques used for the frass odors, includes a majority of dimethyl disulfide and allyl isothiocyanate, a small amount of methyl propyl disulfide heretofore not reported in crucifers, and only traces of dipropyl disulfide (Figure 3). Also, only methylcysteine sulf-oxide, highly abundant, has been identified in crucifers (Tsuno, 1958b; Mae et al., 1971).

If we admit a common biosynthetic origin for the substances identified in the frass of the two species, it thus appears that the proportion of methyl-propyl groups tends to become equal during passage from the plant to the frass. Digestion of the leek moth larvae would preferentially concentrate the substances arising from minor amounts of methyl group precursors in *Allium*, and the digestion of the diamondback moth would preferentially concentrate the substances arising from minor amounts of propyl group precursors in the crucifers. It thus appears that the most abundant precursors cannot be totally transformed, or else that each insect sorts ingested substances differently in its digestive tube.

It would be interesting to determine if these disulfides are specific to the frass of species fed on *Allium* and crucifers by examining the frass odors of other plant-eating larvae. It should also be established if the larvae ingest the precursors directly or, alternatively, substances arising from them. These precursors, in fact, exist only in species closely related to *Allium*, e.g. *Hipheion uniflorum* (Tsuno, 1958a) or crucifers, e.g. *Adenocalymma alliaceum* (Apparao

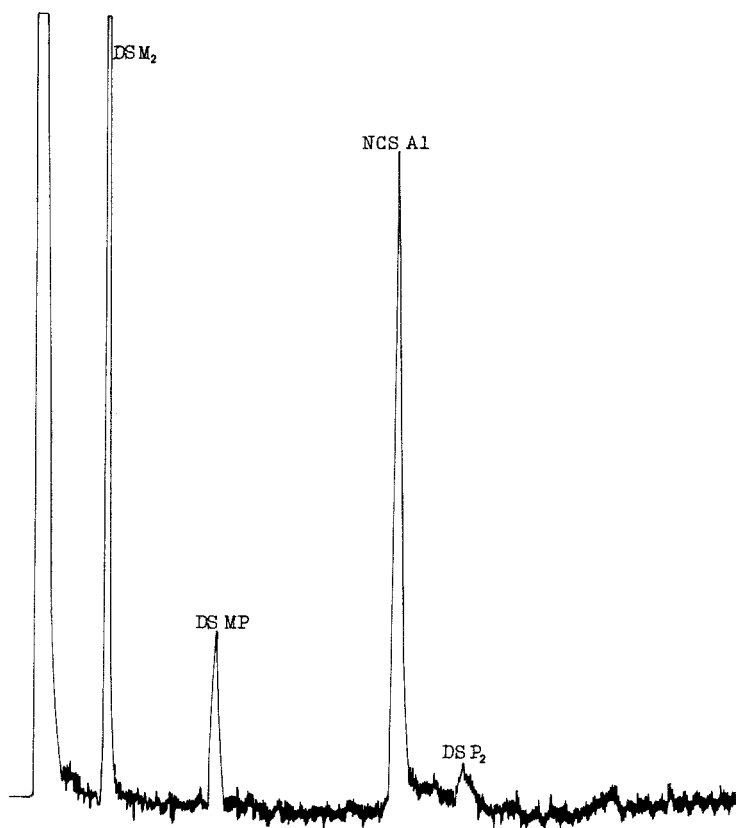


FIG. 3. Gas chromatogram of the cabbage volatiles trapped on Tenax. Column 25 m \times 0.33 mm ID; temperature program: 1°C/min from 80°C to 150°C; on-column injection; FP detector. DS M₂ = dimethyl disulfide; DS MP = methyl propyl disulfide; DS P₂ = dipropyl disulfide; NCS A1 = Allyl isothiocyanate.

et al., 1981), whereas disulfides and especially dimethyl disulfide are sometimes encountered in the odor of plants belonging to other families.

This example of the chemical relationships between host plant, plant herbivore, and entomophage shows the plant origin of the chemical messengers that enable the entomophage to find its host via its frass. This system is possible only when relationships among the various trophic levels are very specific. Another example of this type has been observed in *Camponotus sonorensis* (Elzen et al., 1984).

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RELATIONSHIP OF SEXUAL MATURATION RATE TO RESPONSE OF ORIENTAL FRUIT FLY STRAINS (Diptera: Tephritidae) TO METHYL EUGENOL

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Abstract—Laboratory-reared and wild, fruit-reared adults of the Oriental fruit fly, *Dacus dorsalis* Hendel, were tested for response to methyl eugenol at various ages. Virgin laboratory (2, 4, 6, 8, and 10 days old) and wild (7, 11, 15, 19, and 23 days old) flies were released into an outdoor field cage and trapped over a two-day period. Response of males increased with age as ca. 32% and 22% of laboratory and wild males responded at 2 and 7 days of age, respectively, while ca. 93% of both strains responded at 10 and 23 days of age, respectively. These correspond approximately to the ages at which they reach sexual maturity. Female response did not increase with age and fluctuated between 15% and 29% for the laboratory strain and 10% and 45% for the wild strain. The age-related response profiles, when integrated with sexual maturation curves, indicate that one of the major reasons the male-annihilation technique is effective is because methyl eugenol is able to attract 40–50% of male flies prior to the onset of sexual maturation.

Key Words—*Dacus dorsalis*, Oriental fruit fly, Diptera, Tephritidae, methyl eugenol.

INTRODUCTION

Methyl eugenol is the most effective known lure for attracting male Oriental fruit flies, *Dacus dorsalis* Hendel (Howlett, 1915; Steiner, 1952; Steiner and Lee, 1955; Mitchell et al., 1985). It also attracts many other Dacinae males (Hardy, 1973; Drew and Hooper, 1974, 1981). *D. dorsalis* males feed voraciously on methyl eugenol and usually do so until they die (Steiner, 1952). Steiner et al. (1965, 1970) developed the use of methyl eugenol to successfully

eradicate *D. dorsalis* populations from Rota and other Mariana Islands by air-dropping fiberboard squares saturated with lure-toxicant formulations. This male annihilation method also was used successfully against other *D. dorsalis* populations in the Amami Islands (Ushio et al., 1982) and the Okinawa Islands (Koyama et al., 1984). Presently, the Marianas and Japan are both free of *D. dorsalis*.

Here we report studies on the response to methyl eugenol of various ages of *D. dorsalis* reared in the laboratory on artificial diet (Tanaka et al., 1969) compared with wild flies reared from naturally infested fruits. Also, we compare the present data with sexual maturation data of *D. dorsalis* from Wong et al. (1982).

METHODS AND MATERIALS

Laboratory Oriental fruit flies used in the study had been in culture for ca. 400 generations (at least 32 years, with ca. 12 generations per year). The wild flies were collected as mature larvae from naturally infested papayas, *Carica papaya* L., and guava, *Psidium guajava* L., from the islands of Kauai and Oahu, Hawaii. Two days before eclosion, pupae were dyed with powdered pigments (Day-Glo Arc Yellow, Day-Glo Neon Red, Day-Glo Signal Green, Calco Blue, and Tinopal SFG) using different colors for different ages of flies. The dyed pupae were placed in screened cages (61 × 38 × 37 cm) separated by age group and provided with ICN hydrolyzed protein (Nutritional Biochemical, Cleveland, Ohio), sugar, honey, and water, as needed for survival of newly emerged adults (Keiser and Schneider, 1969). One day after eclosion, flies were separated by sex while immobilized in a refrigerated room (5–7°C) and then transferred to screened cages and held at ambient room condition (average temperature 26.4°C; range 24.6–27.8°C; 66–74% relative humidity under natural daylight supplemented with fluorescent light) until ready for testing. Previous laboratory tests using the same laboratory strain and the same wild population (Wong et al., 1982) had shown differences (both sexes) in physiological ages of the two strains of flies; i.e., sexual maturation of 90% or more was reached under these conditions in ca. 10 days with laboratory-reared flies and in ca. 24 days with wild flies. Therefore, in this study tests were made when laboratory flies were 2, 4, 6, 8, and 10 days old and when wild flies were 7, 11, 15, 19, and 23 days old. The tests were made in two outdoor screened cages (2.5 × 2.5 × 2.5 m). In each of two cages, we released 100 unmated flies of each sex and age, laboratory-reared flies in one and wild flies in the other. Water and sugar were provided in each cage. All releases were made at 0730 hr. A plastic trap (Steiner, 1957) containing a mixture of 99% methyl eugenol and 1% naled by volume in a cotton wick (4 cm long × 2 cm diam.) (Steiner et al., 1965)

was placed in each outdoor cage at 0800 hr, then replaced with another identical baited trap containing methyl eugenol and naled hourly until 1600 hr. The flies become inactive at night and during this time are not attracted to methyl eugenol (Steiner, 1952). These traps were in operation during two days for each release. The test was replicated five times. The numbers of flies trapped for the first day and for the two-day totals were recorded. Temperature and relative humidity ranged from 24 to 27°C and from 68 to 94%, respectively, during testing days. Percentage means were transformed using the arcsine procedure before analysis (Snedecor and Cochran, 1967). Fly age means within laboratory or wild classes were compared with Duncan's (1955) multiple-range test, while male-female differences within each age group were compared with a paired *t* test.

RESULTS AND DISCUSSION

Of the 1677 laboratory-reared and 1487 wild *D. dorsalis* males recovered from traps baited with methyl eugenol, 93.4% and 93.2%, respectively, were taken on the first day. Males of both strains gradually increased their responses to the baited traps with age (Table 1). Roughly 32% and 22% of laboratory and wild males responded on both days (2-day totals) when 2 and 7 days old, respectively, while roughly 93% of both strains responded when 10 and 23 days of age, respectively [$P < 0.05$, Duncan's (1955) multiple-range test]. The response of laboratory females did not vary significantly with age [$P > 0.05$, Duncan's (1955) multiple-range test] and fluctuated between ca. 15% and 29%. The response of wild females was much lower, fluctuating between 4% and 10%, with the response at seven days (4%) being significantly different from the response at 19 days (10%) [$P < 0.05$, Duncan's (1955) multiple-range test]. Although laboratory and wild female mean responses have not been compared directly in Table 1, the magnitude (ca. threefold) of the difference between strains clearly supercedes any effect due to possible difference in physiological age and contrasts sharply with the very similar responses for the males of each strain. The responses of males and females are significantly different from each other at all ages for both strains ($P < 0.05$, *t* tests) (Table 1).

As shown in Figure 1, males of both strains began to respond to methyl eugenol before any were sexually mature. Indeed, 40-50% of males of both strains responded before their respective ages of earliest mating. Also shown in Figure 1 are mating data from Wong et al. (1982), which provide a comparison of mating response with male attractancy. Best-fitting linear regressions for percentage response (*Y*) of laboratory-reared and wild males of different ages (*X*) to traps baited with methyl eugenol are: $Y = 5.6(X) + 19.8$ ($r = 0.995$) and $Y = 2.8(X) + 6.1$ ($r = 0.992$), respectively, and for laboratory-reared and wild males mated at different ages are: $Y = 10.0(X) - 22.4$ ($r = 0.987$) and $Y =$

TABLE 1. RESPONSE OF LABORATORY-REARED AND WILD *Dacus dorsalis* OF DIFFERENT AGES TO TRAPS BAITED WITH METHYL EUGENOL AND 1% NALED IN OUTDOOR CAGES, HONOLULU, HAWAII

Strain	Age (days)	No. flies recovered on 1st day (mean \pm SEM)		Total flies recovered on 1st and 2nd days (mean \pm SEM)		T test probability $ H_0: \bar{X}(\sigma\sigma') = \bar{X}(\varphi\varphi) ^c$
		Males	Females	Males	Females	
Laboratory	2	23.6 \pm 2.2 ^a d ^b	10.0 \pm 2.2 a	31.8 \pm 3.5 d	15.4 \pm 2.8 a	0.0069
	4	46.8 \pm 3.8 c	22.4 \pm 5.4 a	53.2 \pm 4.0 c	26.8 \pm 4.8 a	0.0030
	6	68.0 \pm 7.9 b	16.6 \pm 4.8 a	73.0 \pm 6.7 b	18.6 \pm 5.0 a	0.0003
	8	82.2 \pm 2.8 a	24.2 \pm 8.6 a	83.6 \pm 3.5 ab	25.6 \pm 8.5 a	0.0013
	10	92.6 \pm 1.0 a	26.2 \pm 10.4 a	93.8 \pm 1.2 a	28.8 \pm 10.9 a	0.0003
Wild	7	17.0 \pm 4.8 d	3.4 \pm 0.9 b	22.0 \pm 5.0 d	4.0 \pm 1.0 b	0.0076
	11	37.4 \pm 5.4 c	4.8 \pm 1.5 ab	41.6 \pm 4.9 c	5.4 \pm 1.7 ab	0.0010
	15	61.8 \pm 4.0 b	4.8 \pm 1.0 ab	65.6 \pm 4.4 b	6.4 \pm 1.8 ab	0.0001
	19	71.2 \pm 2.3 b	8.0 \pm 1.8 a	75.6 \pm 2.5 b	10.0 \pm 1.7 a	0.0001
	23	89.8 \pm 1.6 a	6.6 \pm 1.6 ab	92.6 \pm 1.3 a	8.4 \pm 1.7 ab	0.0001

^a Values are means (\pm SEM) based on five replicates of 100 flies/sex per strain released into cages.

^b Means followed by the same letter within the same sex and strain are not significantly different [$\alpha = 0.05$; Duncan's (1955) multiple range test].

^c T test probability comparing male and female means for same age and strain. Null hypothesis: mean number of males = mean number of females.

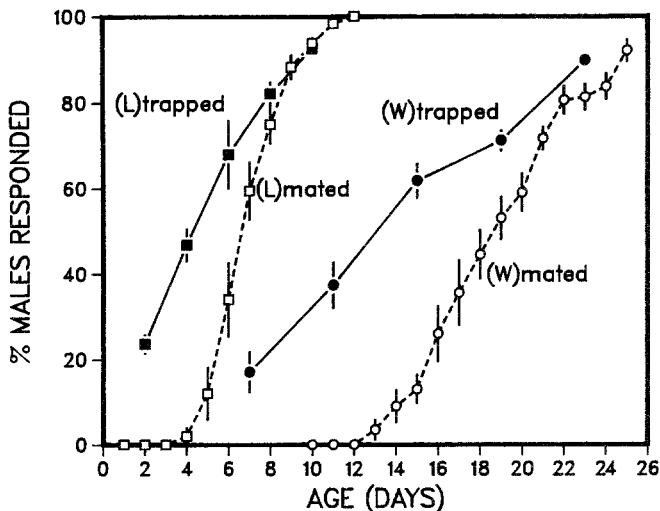


FIG. 1. Response of laboratory-reared (L) and wild (W) *Dacus dorsalis* males of different ages to traps baited with methyl eugenol and 1% naled in outdoor cages, Honolulu, Hawaii. Values shown are arcsine transformed percentage means for five replicates (trapped) and 10 replicates (mated). Lines at each point represent ± 2 standard errors. Data on mating of laboratory-reared and wild flies obtained from Wong et al. (1982).

5.2(X) - 55.4 ($r = 0.993$), respectively. When males were older (8-day-old laboratory and 23-day-old wild males), responses to the lure matched sexual maturation rates for each strain. Mating percentages of the following five age groups were significantly different from each other for the lab strain: 1-3 days, 4-5 days, 6 days, 7-8 days, and 9-11 days [$P < 0.05$, Duncan's (1955) multiple-range test]. For the wild strain, five age groups were significantly different from each other: 12-13 days, 14-15 days, 16-17 days, 18-20 days, and 21-24 days. The results differed from those of other workers. We found that significant attractancy to methyl eugenol by male *D. dorsalis* can occur prior to sexual maturation; others found that attraction of *D. dorsalis* (Umeya et al., 1973; Ito and Iwahashi, 1974) and *Dacus opiliae* Drew & Hardy (Fitt, 1981) was correlated with sexual maturity. However, Steiner (1952) reported that sexually immature *D. dorsalis* (as young as 1 day old) males responded to methyl eugenol.

Of the total males recovered in traps, most flies were taken within the first 3 hr after they were released, 89 and 92% for laboratory-reared and wild males, respectively. However, if flies had been released in the afternoon instead of in the morning, males would probably have responded just as well because Steiner (1952) found that methyl eugenol is effective at dawn and dusk as well as throughout the day but does not attract male flies at night. In the field, Naka-

gawa and Urago of this laboratory (unpublished data) found that wild *D. dorsalis* males had two peak response periods, one early in the morning and the second in the mid-afternoon.

The possible existence of an insensitive strain of *D. dorsalis* males to methyl eugenol has been reported by Ito and Iwahashi (1974). In Chichi Jima of the Ogasawara Islands of Japan, researchers selected a strain of male flies that was recaptured in a frequency consistently lower than that for control flies. Because of this finding, the eradication program used against *D. dorsalis* in the Ogasawara Islands involved a combination of male annihilation and sterile insect release methods (Habu et al., 1980). However, eradication of *D. dorsalis* in the Okinawa Islands was by the male annihilation method alone (Koyama et al., 1984), suggesting that at least in Okinawa, *D. dorsalis* was completely sensitive to methyl eugenol.

Females were less responsive than males to traps baited with methyl eugenol. However, significant numbers of mated and unmated flies (576 laboratory-reared and 171 wild females) were caught. The responding females on the first day of testing (average of 86% for all ages) were probably all virgins because they would not be able to mate until near sunset. Kobayashi et al. (1978) found that *D. dorsalis* mates just before dusk. These results differ sharply from those of Steiner (1952), who found that only virgin *D. dorsalis* females were slightly responsive to methyl eugenol in the laboratory. We also found that the proportions of females responding were much higher for both strains at all ages than reported by Steiner (1952), suggesting that the experimental design contributed to higher than expected female yield, in proportion to male yield. During large-scale pilot tests against *D. dorsalis* in the Bonin and Mariana Islands between 1960 and 1963, sexually mature virgin females were attracted to, and killed by, poisoned methyl eugenol placed on baited stations or in traps. The attraction occurred only when populations of males were almost depleted, as indicated by catches of < 1 male per trap per day (Christenson, 1963; Steiner et al., 1965).

One of the major reasons the male annihilation technique is highly effective in eradicating *D. dorsalis* populations (Steiner et al., 1970; Koyama et al., 1984) is because 40–50% of the male flies are attracted to methyl eugenol-toxicant mixtures and killed before any become sexually mature.

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PATTERNS OF DEPOSITION OF URINE CONTAINING
CHEMOSIGNALS THAT AFFECT PUBERTY AND
REPRODUCTION BY WILD STOCK MALE AND
FEMALE HOUSE MICE (*Mus domesticus*)

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Abstract—House mice release chemosignals in their urine that influence the timing of puberty and reproductive condition in conspecific females. These experiments tested the possibility that mice depositing urine containing chemosignals that affect puberty and reproduction do so differentially with respect to urine cues from conspecifics. Mice were tested in cages containing samples of urine or water on cotton in wire mesh capsules protruding from the cage floor. Their urine deposition patterns were recorded on squares of filter paper positioned below the cages. Males deposited more urine than females housed in groups, estrous females, diestrous females, or prepubertal females. All groups of mice deposited urine in a nonrandom fashion with regard to urine cues from conspecifics. Male mice deposited more urine near locations previously soiled by females than near water or other males. Grouped females deposited more urine near male urine cues and avoided depositing urine near urine from other group-housed females. Both estrous and diestrous female mice deposited more urine near males than near other urine cues or water, possibly to attract mates. Prepubertal females avoided depositing their urine near male urine and urinated more near urine from grouped females than near other urine types or water. Young females may be avoiding possible male mates until they have attained puberty. This avoidance behavior may enhance the long-term reproductive success of the females that otherwise might mature and mate at too young an age.

Key Words—House mice, urinary chemosignal, puberty, reproductive strategy, urine deposition, estrous, diestrous, mouse density, *Mus domesticus*.

INTRODUCTION

The age of first vaginal estrus in female house mice can be altered by social cues and urinary chemosignals from conspecifics (Vandenbergh, 1983; Drickamer, 1986a). Housing young females with adult male mice or treating young females with urine from male mice, female mice in estrus, and female mice that are pregnant or lactating all result in accelerated onset of puberty (Vandenbergh, 1967, 1969; Colby and Vandenbergh, 1974; Drickamer and Murphy, 1978; Drickamer and Hoover, 1979; Drickamer, 1986b). Housing young females with other young or adult females, or treating them with urine from grouped females results in delays in the onset of puberty (Vandenbergh, et al., 1972; Drickamer, 1974, 1977). The urinary chemosignals that influence sexual development have been explored from a variety of perspectives including timing effects, hormonal involvement for donors and recipients, density and dominance effects, volatility, doses, etc. (see Vandenbergh, 1983; Drickamer, 1986a).

Urine cues from conspecifics also influence the reproductive condition of adult female mice, including cues from males (Whitten, 1957; Bruce, 1960; Parkes and Bruce, 1962; Kimura, 1971) and other females (Whitten, 1959; Hoover and Drickamer, 1978). These phenomena involving the reproductive physiology and behavior of adult females have been explored from a variety of perspectives, including many of those mentioned above with regard to chemosignal effects on puberty (see Bronson, 1979; Marchlewska-Koj, 1983; McClintock, 1983; Vandenbergh, 1983, for reviews).

Urine marking by mice has been studied both in the laboratory and under natural conditions (Bronson, 1976; Hurst, 1987) and with respect to identification of groups or individuals (Jones and Nowell, 1974; Cox, 1984), the tendency to investigate novel surroundings (Maruniak et al., 1974; Matthews, 1980), urination as a social response (Reynolds, 1971), differential urination patterns dependent upon social rank in males (Desjardins et al., 1973), and the possible use of urine cues as territorial boundary marks (Mackintosh, 1973; Harrington, 1976). The urinating "posts" described by Welch (1953) and later reported by Hurst (1987) may function with respect to territory, although Hurst (1987) has suggested that the posts may also be related to orientation and detection of novel stimuli in the environment. Urine cues, in the form of posts or surface deposits may therefore serve several functions simultaneously.

Mechanisms underlying these urine deposition behaviors and their significance have been investigated with respect to sex steroids (Maruniak et al., 1975; Kimura and Hagiwara, 1985) and genetics (Beauchamp et al., 1986).

Using a laboratory stock of mice (C3M/Me-Mg), Wolff and Powell (1979) found that female mice made significantly more urine spots on filter paper dur-

ing proestrus and estrus than at other stages of the cycle, with a dramatic decline after estrus. In a laboratory study involving circular arenas, male mice produced more urine spots that were more regularly dispersed, whereas female mice produced fewer urine deposits with more clumped distribution patterns (Powell and Wolff, 1982). Further, Wolff and Powell (1984) reported that male mice tended to urinate more in locations that had been previously marked with urine; they tended to deposit more urine spots on areas with cues from proestrous and ovariectomized females than areas containing cues from other females. Females deposited more urine at the time of estrus than at other stages of the estrous cycle. Females deposited more urine near urine from intact than castrated males.

One topic pertaining to the puberty-influencing urinary chemosignals that has not been investigated concerns the patterns of urine deposition by male and female mice with respect to cues from conspecifics. Is it possible that mice may be depositing their urine differentially with respect to cues from conspecifics? If so, there could be significant consequences for the effects of the chemosignals in the urine on physiological mechanisms controlling sexual maturation in young females and the reproductive condition of adult females.

The two experiments performed here were designed to assess the following questions. (1) Do mice differentially deposit their urine depending upon what other urine cues are present in the environment? (2) Do mice deposit their urine in a manner that might enhance the physiological effects of any puberty-influencing chemosignals or chemosignals affecting reproductive condition of adult females contained in that urine?

METHODS AND MATERIALS

General Methods. The mice used for these experiments were third and fourth generation descendents of a stock of wild house mice (*Mus domesticus*) trapped near West Simsbury, Connecticut. All mice were maintained in opaque polypropylene cages measuring 28 × 15 × 15 cm deep with fitted wire lids containing food and water receptacles. A bedding of ground wood shavings was changed once each week. Pregnant female mice were given cotton squares for making nests. Wayne Lab Blox and water were provided ad libitum to all mice throughout the experiments, except for the 22-hr periods that mice were in the test cage. All colony cages and the test apparatus were housed in the same two rooms throughout the 18 months of the study. These rooms were maintained at 21–25°C and 30–60% relative humidity on a 12-hr dark: 12-hr light daily regimen with overhead fluorescent lights on from 0600 to 1800 hr.

Mice used for collection of urine and those used as test subjects were produced by mating adult male and female wild stock mice for a total of 84 pairs

producing sufficient litters for these experiments. Mice were weaned from their parents at 25 days of age and housed in like-sex groups or individually until needed. Details of housing arrangements are specified with each experiment.

Experiment I. The first experiment was designed to assess the urine deposition patterns of wild stock house mice presented with a choice between a conspecific urine cue and water.

Five different groups of test subjects were used: (1) sexually experienced adult females (ages 90–145 days) grouped with eight mice per cage for at least 30 days prior to testing; (2) sexually experienced adult males (ages 90–185 days) housed individually for at least 20 days prior to testing; (3) sexually experienced adult females (ages 85–130 days) in vaginal estrus on the day of testing; (4) sexually experienced adult females (ages 85–130 days) in vaginal diestrus on the day of testing; and (5) prepubertal females (ages 35–45 days) weaned at 25 days of age and housed individually until testing. For groups (3) and (4), daily vaginal smears were taken to determine which females were in estrus and diestrus.

For each experimental group, 96 mice were tested. The test apparatus consisted of a 0.5×0.5 -m stainless-steel cage with solid walls, a wire mesh floor, a wire mesh top, and short legs to suspend the cage 3 cm above the table top. The mesh in the floor contained 4 squares/cm². For each trial, two previously unused squares (3×3 cm) of fine screen mesh (25 squares/cm²) were pushed upward through the floor in diagonally opposite corners of the cage. A ball of sterile cotton was placed inside this pocket of fine screen. A syringe was used to inject 0.2 cc of urine or water into the center of each cotton ball. A flip of a coin determined which square was injected with urine and which with water for each separate trial. A piece of filter paper measuring 0.5×0.5 m was positioned directly below each test cage.

Using this apparatus, the urination patterns of mice were tested in the presence of water vs. urine from (a) adult males, (b) grouped adult females, (c) estrous females, (d) diestrus females, (e) prepubertal females, and (f) against water. The mice used for collecting the urine cues were the same groups of mice used as test subjects. No mouse was handled for urine collection for two days prior to or one day after being used as a test subject in the apparatus. Urine was collected by holding each mouse over a Petri dish and gently squeezing the flanks. Fresh urine was collected each day for use in the test cages. This required collecting urine from 15–20 mice of each type. Separate Petri dishes and syringes were maintained for each urine type. Sixteen mice of each treatment group were tested for each combination of test stimuli.

The test procedure involved placing a mouse in the apparatus for 22 hr after having placed urine on the cotton balls and filter paper under the cage. The filter paper was marked with appropriate information about the test subject

and the nature and location of the urine and water test stimuli. At the end of the test period, the mouse was removed and returned to its original cage.

The pieces of filter paper were scanned with an ultraviolet light (Desjardins et al., 1973) and all urine spots were marked. The marked filter paper was then placed on top of a grid on a light table. The scoring grid measured 20×20 squares (each grid square was $2.5 \times 2.5 = 6.25 \text{ cm}^2$). Three types of scoring data were obtained: (1) the total number of squares in the pen containing any urine (maximum = 400), (2) the number of squares within a 3-cm radius surrounding each stimulus source (maximum for each source = 36), and (3) the pattern of distribution of urine throughout the test cage was measured by dividing the grid into 25 sections of four squares each. A mouse that deposited urine in all sections of the test cage could receive a score of 25. Data from the first dependent variable were used to assess possible differences in total squares marked for the different test groups. They were also used in conjunction with the second dependent variable to assess the percentage of squares containing urine near the stimuli in relation to the total squares containing urine. The third dependent variable was used to assess the overall distribution of urine deposition in the test cage for each treatment group. One-way analyses of variance were used to determine significant patterns of variation across the treatment groups for each dependent variable.

The second dependent variable was used to assess possible differential responses within each of the five test groups to the two stimuli presented to each test mouse. To do this, a ratio of squares marked near the urine source to the squares marked near the water source was computed for each trial. Because of problems with variance heterogeneity and skewness, we then took the natural log of each ratio (Lenington, 1983; Egid and Lenington, 1985); we would expect to find ratios greater than 0 if the test mice were depositing more urine near the stimulus cotton that contained urine and ratios less than 0 if the test mice were depositing more urine near the water cue. For the test situation with water in both stimulus cotton balls, the ratio should not differ significantly from 0. For each test group, these ratios were subjected to a one-way analysis of variance and then Duncan's new multiple-range test with $\alpha = 0.02$.

Experiment II. The second experiment was designed to assess the possible differential urine deposition patterns for three type of mice: males, grouped females, and prepubertal females when mice were presented with pairs of urine stimuli in the two cotton balls used in the test apparatus for each trial. The five test pairs of urine used were: (a) males vs. grouped females, (b) males vs. estrous females, (c) males vs. prepubertal females, (d) estrous females vs. diestrous females, and (e) estrous females vs. prepubertal females.

The apparatus and procedures for each trial were identical to those in experiment I. For these trials, 75 different mice were tested from each of the

three groups with 15 mice used for each of the five different pairs of urine stimuli injected into the cotton balls. One-way analyses of variance were performed within each pair of urine stimuli followed by post-hoc tests of ratios of squares containing urine within the 3-cm radius of each stimulus.

RESULTS

Experiment I. Adult males deposited urine in more total squares throughout the test cage and in more squares within the 3-cm radii around the stimuli on average than mice in the other groups (Table 1); the other groups did not differ from one another for either dependent variable. The same pattern of significant differences was true for measures of the proportion of squares containing some urine that were within the 3-cm radii and the total number of squares (of a possible maximum of 72 for the two stimulus circles combined) that contained urine (Table 1). For the separate measure of the total distribution pattern of urine in the test cage, there were no significant differences across the five test groups (Table 1).

Grouped female mice (Figure 1A) deposited urine in more squares near male urine than near water, but deposited urine in more squares near water than

TABLE 1. URINE DEPOSITION PATTERNS BY TEST MOUSE TYPES

Test mouse type	Total squares in pen ^a	Squares within 3 cm of sources ^b	Percentage of total squares within 3 cm ^c	Number of squares within 3 cm ^d	No. of quadrants ^e
Adult males	68.4 (3.8) ^{b,f}	41.7 (1.3) ^b	53.8 (3.1) ^b	39.9 (2.3) ^b	15.8 (0.8)
Grouped adult females	41.4 (2.6) ^a	18.9 (0.9) ^a	41.2 (2.3) ^a	25.7 (1.6) ^a	14.6 (1.0)
Adult females in estrus	45.6 (3.4) ^a	21.6 (2.5) ^a	42.1 (2.8) ^a	23.2 (1.4) ^a	14.4 (1.0)
Adult females in diestrus	37.5 (2.1) ^a	17.8 (1.3) ^a	40.7 (2.7) ^a	23.1 (1.9) ^a	14.6 (0.9)
Prepubertal females	39.4 (2.5) ^a	15.0 (1.1) ^a	36.1 (2.1) ^a	21.1 (1.6) ^a	14.2 (0.7)
<i>F</i> (<i>df</i> = 4,475)	28.97	18.18	7.07	22.04	0.15
Probability	<0.001	<0.001	<0.01	<0.001	n.s.

^a Mean numbers of squares (\pm SEM) containing at least some urine out of a possible total of 400 squares throughout the test pen.

^b Mean number of squares containing at least some urine within a circle of 3 cm to the two urine sources, out of a total of 36 possible squares.

^c Percentage of squares containing at least some urine within the 3 cm radius of the two stimulus sources.

^d Percentage of the total possible 72 squares around the two stimuli that contained urine.

^e Total number of quadrants (maximum = 25) throughout the pen that contained urine.

^f Those means in the same vertical column not marked with the same superscript letter are significantly different at $P < 0.02$ by Duncan's new multiple-range test. $N = 96$ mice/treatment.

near urine from grouped females. The responses of grouped females to the other urine-water pairs did not differ from the expected value of 0.

Adult male mice (Figure 1B) deposited urine in significantly more squares near urine cues from females than water. The response of males to male urine did not differ from the expected value of 0.

The patterns of responses by estrous and diestrous females were similar (Figures 1C, D). These mice deposited urine in significantly more squares near males than water, but deposited urine in more squares near water than urine from grouped females. Their responses to the other urine-water pairs did not differ from the expected value of 0.

Prepubertal females (Figure 1E) deposited urine in significantly more squares near the water cue when tested against male urine, but urinated in more squares near the grouped female urine than the water. There was an overlapping pattern of responses by the prepubertal females to the other urine-water pairings, but none of these tests resulted in significant differences from the expected value of 0.

Experiment II. When tested with male urine vs. grouped female urine, both male and prepubertal female test subjects deposited urine in significantly more squares around the urine cue from grouped females, whereas grouped females as test subjects deposited urine more often near the male urine cue (Figure 2A). When the test pair consisted of either male urine vs. estrous female urine or male urine vs. prepubertal female urine, a pattern of results similar to that seen for male urine vs. grouped female urine was observed (Figures 2B, C). For the test pairs involving estrous female urine vs. diestrous female urine and estrous female urine vs. prepubertal female urine, male mice deposited urine in significantly more squares near the urine from estrous females, and both grouped females and prepubertal females exhibited urination patterns that did not differ from the expected value of 0 (Figures 1D, E).

DISCUSSION

These experiments provide the bases for three conclusions: (1) In the test situation used, male mice deposited urine in more total squares than other test groups, but did not differ from the other treatments in terms of the overall dispersion pattern of urine deposition. (2) Male mice deposited significantly more urine near stimulus sources than other types of test mice. (3) All mice appeared to differentially deposit urine near specific urine cues in at least some test situations.

Male mice marked more than the other types of mice tested. This finding is in agreement with other published data (Reynolds, 1971; Maruniak et al.,

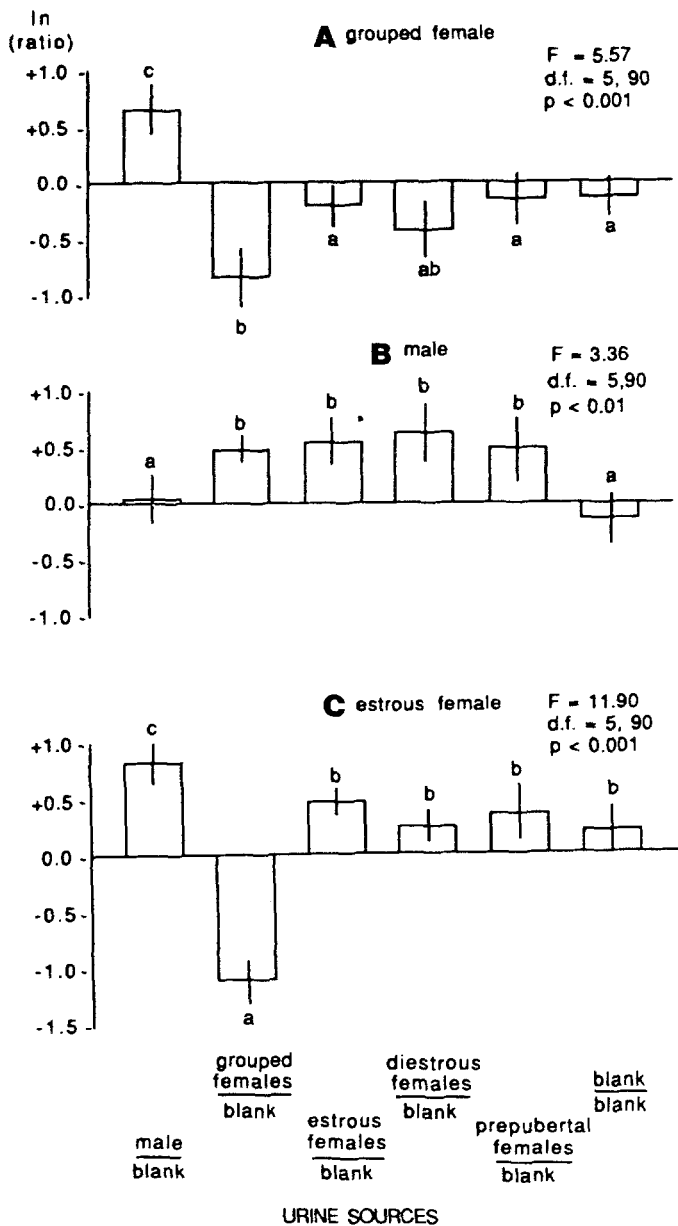
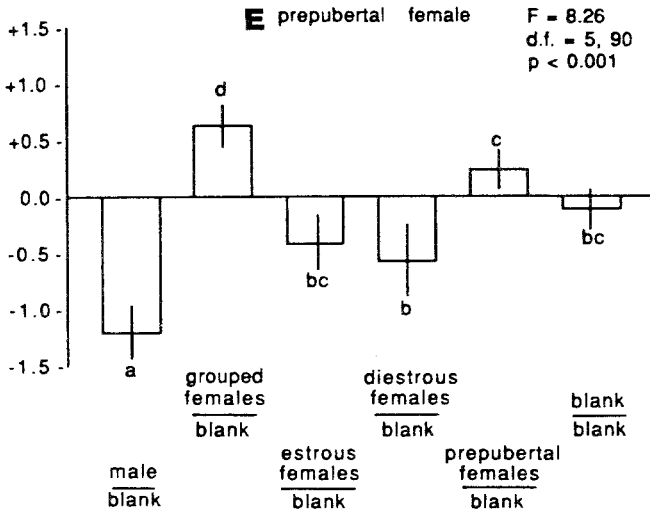
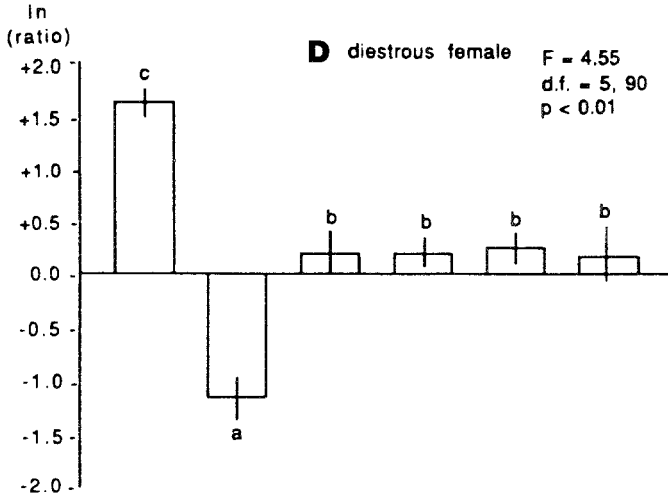


FIG. 1. Means of natural logarithms of ratios for numbers of grid squares containing urine in 3-cm-diameter circles surrounding two urine sources (± 1 SEM = vertical lines). Each of five types of test mice: (A) grouped females, (B) males, (C) estrous females, (D) diestrous females, and (E) prepubertal females, were tested with five types of urine vs. water (designated as "blank"), shown at the bottom of the figure. A control test



URINE SOURCES

involved water at both stimulus locations. Sixteen mice of each type were tested with each of the six treatment pairings. The F ratios from one-way analyses of variance and associated probabilities are shown for each test mouse type. Within each experiment, those bars not marked with the same letter are significantly different at the 0.02 level using Duncan's new multiple-range test. A mean ratio of 0 denotes that approximately equal proportions of grid squares were marked around each of the two stimulus sources; significant deviations above or below the line indicate greater numbers of grid squares containing urine near the urine cue or water respectively.

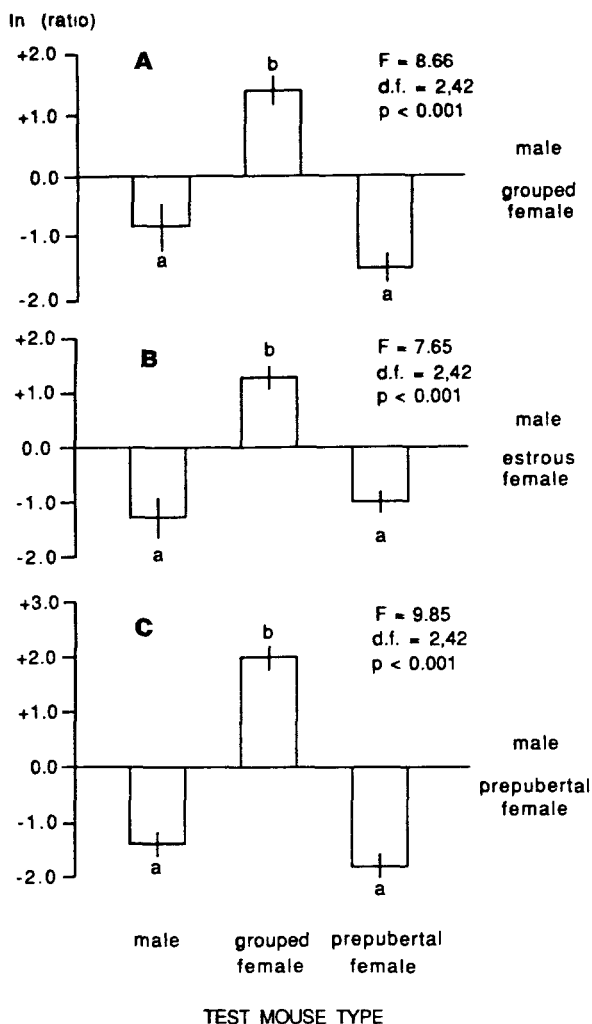


FIG. 2. Means of natural logarithms of ratios for numbers of grid squares containing urine in 3-cm-diameter circles surrounding two urine sources (± 1 SEM = vertical lines). Male mice, grouped female mice, and prepubertal female mice (test mouse type) were each tested using five different pairs of urine stimuli: (A) male urine vs. grouped female urine, (B) male urine vs. estrous female urine, (C) male urine vs. prepubertal female urine, (D) estrous female urine vs. diestrous female urine, and (E) estrous female urine vs. prepubertal female urine. Fifteen mice of each type were tested with each of the five pairs of urine sources. Analyses were run for each of the five urine combinations tested; the F ratios from one-way analyses of variance and associated probabilities are shown. Within each urine combination, those bars not marked with the same letter are significantly different at the 0.02 level using Duncan's new multiple-range test. A mean ratio of 0 denotes that approximately equal numbers of grid squares were marked around each of the two stimulus sources; significant deviations above or below the line indicate greater numbers of squares marked near the designated urine source.

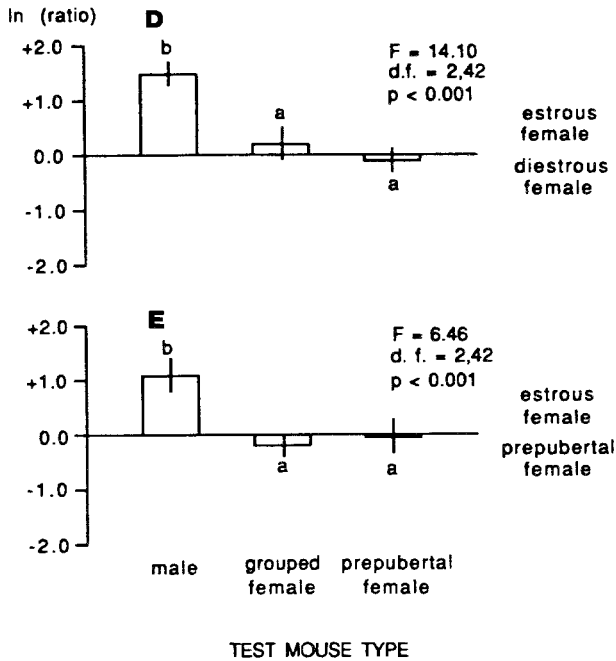


FIG. 2. Continued.

1975; Powell and Wolff, 1982; Kimura and Hagiwara). The finding in experiment I that males disperse their urine throughout a test cage is also in agreement with previous reports (Powell and Wolff, 1982), although these data may be viewed as contrasting with earlier findings reported for *Mus* (Desjardins et al., 1973) that indicated specific patterns of urine deposition restricted in overall distribution to specific areas of a test cage. The present data also indicate that the stock of wild mice used for these investigations does not counter-mark as frequently as has been reported by Wolff and Powell (1984). Hurst (1987) also comments on the failure to find extensive counter-marking by male mice in her natural setting. Males consistently deposited more urine near cues from females in both experiments. In experiment II they deposited more urine near urine from estrous females than either diestrous or prepubertal females. It would appear that male mice deposit their urine in a fashion that could enhance the onset of puberty in young female mice encountering the urine and in a manner that would affect the reproductive physiology of conspecific adult females to enhance their estrous cycles; in both instances the effects would be to increase the number of potential mates available for reproduction.

Grouped female mice clearly avoided the urine of other grouped females in terms of their patterns of urine deposition. They also deposited less urine

near estrous or diestrous females than near other cues in paired choice situations. They provided neutral responses toward urine from prepubertal females. The most significant reaction of grouped females in the test situation was to deposit significantly more urine near male urine when possible. It thus appears that females may be (1) avoiding the odor of other grouped females, remaining away from possible effects of the urinary chemosignal from females housed under high-density conditions that delays the onset of puberty (Vandenbergh et al., 1972; Drickamer, 1977), and (2) leaving urine cues at sites they associate with males, signaling their presence as possible mates. This last finding concurs with the report by Wolff and Powell (1984) regarding female counter-marking on sites previously marked by males.

Estrous and diestrous female mice responded in similar ways in the test situations used here. They consistently avoided grouped female urine and left significant urine deposits near urine from males. Their responses to prepubertal female urine and to urine from estrous and diestrous females were neutral in both experiments. As with the grouped females, it is possible these females are avoiding urine that could retard their reproductive condition while leaving scent cues near sites associated with males to indicate their presence as possible mates. Data from experiment I indicate that both estrous and diestrous females marked the same total area of the test cage. This may be in contrast to the report by Wolff and Powell (1979) that females in proestrus and estrus deposited more urine spots than females at other stages of the estrous cycle. It is possible that the methods of measuring urine deposition are different enough in these two studies that the comparison is not valid. Also, these experiments measured estrous and diestrous stages only of wild stock mice, whereas Wolff and Powell (1979) were concerned with all stages of the cycle and used a laboratory strain.

Lastly, prepubertal females avoided male urine and tended to deposit significantly more of their urine near urine from grouped females. This conclusion is supported by data from both experiments. The responses of prepubertal females to the other urine cues were generally neutral. The responses by prepubertal females to grouped female and male urine are directly opposite to the responses recorded for other types of females tested. Why should prepubertal females behave so differently than adult females? One possible explanation lies in a recent report regarding the long-term consequences of early and late puberty in female mice with respect to reproduction (Drickamer, 1988). Young female mice that are accelerated in their sexual development have a higher mortality rate early in life, produce fewer litters, and have, on average, smaller litters than females that are delayed in attaining puberty. Thus, by avoiding contact with male urine and not exposing themselves to a chemosignal that could accelerate their sexual development, young females may be escaping some negative consequences. In addition, as has been suggested by Bronson (1979), young females may avoid male urine containing the puberty-accelerating chemosignal until after dispersal.

Both male and female mice exhibit active patterns of urine deposition in the presence of other urine cues from conspecifics. Some of these observed patterns suggest that female mice may be regulating their reproductive physiology by exposing or not exposing themselves to particular urine stimuli. Other data support the conclusion that mice of both sexes will, under appropriate conditions, seek to mark in the same area as that used by a conspecific of the opposite sex, providing the basis for exchanging information on the whereabouts of potential reproductive partners.

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DESCRIPTION OF SEXUAL BEHAVIORS OF *Drosophila*
rajasekari
The Role of (Z,Z)-7,11-Heptacosadiene

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Abstract—The courtship behaviors and cuticular hydrocarbons of *Drosophila rajasekari* are described. Sexually mature males orient, tap, follow, vibrate their abdomens, extend and vibrate their wings, and attempt copulation during courtship. They perform these behaviors in response to immature and mature *D. rajasekari* of both sexes, and their courtship activities are facilitated by light. The predominant cuticular hydrocarbon found in both sexes is (Z,Z)-7,11-heptacosadiene (HCD), a compound known to be used as a courtship-stimulating sex pheromone by another fruit fly, *D. melanogaster*. Therefore, it is not surprising that *D. melanogaster* males actively court both males and females from the *D. rajasekari* stock. However, HCD is apparently not used by *D. rajasekari* as a courtship-stimulating pheromone since mature *D. rajasekari* males do not court *D. melanogaster* females, which produce large quantities of HCD.

Key Words—Courtship, pheromones, (Z,Z)-7,11-heptacosadiene, *Drosophila rajasekari*, *Drosophila melanogaster*, Diptera, Drosophilidae.

INTRODUCTION

Since Sturtevant's classic paper in 1915, many studies have concentrated on the cues that mediate sexual behavior in *Drosophila*. In the most commonly studied situation, that of a *D. melanogaster* male courting a virgin conspecific female, the male performs an elaborate series of courtship behaviors that are elicited by visual, olfactory, and tactile cues provided by the female (reviewed by Tompkins, 1984). In this species, the major olfactory cue is a 27-carbon alkadiene

known as (Z,Z)-7,11-heptacosadiene (HCD) (Antony and Jallon, 1982; Antony et al., 1985). This compound is produced in large quantities by mature females but is not found in males (Antony and Jallon, 1982). It is this strong sexual dimorphism for a courtship-stimulating pheromone that causes *D. melanogaster* females to be sexually attractive while mature *D. melanogaster* males are not.

Drosophila rajasekari was first described (Sreerama Reddy and Krishna Murty, 1968) from collections made in Mysore, India, and, based on morphological features, was assigned to the *suzukii* subgroup of the *melanogaster* species group in the subgenus *Sophophora*. Our preliminary chemical analysis of these flies indicated that little sexual dimorphism existed in terms of their cuticular hydrocarbons. In addition, we were interested to discover that the major cuticular compound produced by *D. rajasekari* males and females was HCD.

D. rajasekari thus represents a species in which the major cuticular hydrocarbon is HCD (like *D. melanogaster* females) but, unlike the situation in *D. melanogaster*, they show no gross sexual dimorphism for cuticular hydrocarbons. Accordingly, we set out to analyze the sexual behaviors of *D. rajasekari* and to determine whether or not they also use HCD as a courtship-stimulating pheromone.

METHODS AND MATERIALS

Stocks. The *D. rajasekari* stock, strain 14023-0361, was obtained from the National Drosophila Resource Center in Bowling Green, Ohio. The *D. melanogaster* stock, Canton-S strain, was obtained from L. Tompkins at Temple University in Philadelphia, Pennsylvania. Both stocks were maintained in mass culture on Formula 4-24 Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, North Carolina) under a 16:8 light-dark cycle at ambient laboratory temperature. All flies were collected within 3 hr of eclosion and separated by sex under CO₂ anesthesia. Males were stored individually, while females were stored in groups of 2-20 in vials containing media.

Extraction and Analysis of Cuticular Hydrocarbons. Cuticular compounds were extracted from individual flies by soaking them in 100 μ l of hexane for 1 min (Scott, 1986). Extracts from large groups of flies were made by soaking the flies in hexane for 24 hr, followed by chromatography over silica eluted with hexane (Bartelt and Jackson, 1986). Hydrocarbons were separated by degree of unsaturation on a AgNO₃ HPLC column (Bartelt et al., 1986a). Alkanes and alkenes were eluted with 5% toluene in hexane, and alkadienes were eluted with 25% toluene in hexane. Gas chromatography (GC) was performed on a 30-m DB-1 capillary column programmed from 150 to 300°C at

10°/min, and a 30-m DB-225 capillary column programmed from 150 to 200°C at 5°/min. A 30-m DB-5 capillary column was utilized for introduction into a VG-MM16 mass spectrometer. Each peak was characterized by its equivalent chain length based on the comparison of the retention time of the peak with the retention times of an internal standard of paraffin hydrocarbons, area integrated on a Hewlett Packard 3380A integrator, mass spectra of saturated hydrocarbons (Blomquist et al., 1987), and mass spectra of the dimethyl disulfide reaction products (Nichols et al., 1986; Vincenti et al., 1987) for the unsaturated hydrocarbons.

Behavioral Assays. Behavioral assays were performed between 9:00 AM and 9:00 PM at room temperature. For each assay, a "test male" and a "sex object" (a male or female fly used to elicit courtship from the test male) were aspirated without anesthesia into a cylindrical plastic chamber (vol ca. 0.2 cm³) and observed for 500 sec. During each observation period, the courtship index (CI), the percentage of the observation period that the test-male spent performing any of the courtship behaviors toward the sex object (Tompkins et al., 1980), was recorded.

To determine the age at which *D. rajasekari* males become sexually mature, 6-hr- to 6-day-old males were tested with females of the same age. Flies tested at 6 hr and 12 hr were collected within 15 min of eclosion. The CI and the behaviors performed by each male were recorded, and males were judged to be sexually mature at an age at which they performed all of the sexual behaviors, copulated with females, and had CIs as high as males that were one day older (see McRobert and Tompkins, 1987).

Sexually mature males were then tested with *D. rajasekari* males and females of various ages, as well as mature (3 to 5 days old) *D. melanogaster* females, to identify sexually attractive flies. In tests in which two males were tested together, one of the males had the distal tip of one wing removed as a marker. In half of these tests, the courting male was marked; in the other half, the "sex object" male was marked.

To determine whether *D. rajasekari* males were able to perform courtship in the dark or whether *D. rajasekari* males and females were attractive in the dark, flies were tested under a red photographic safelight. Since *Drosophila* do not respond to red light (McEwen, 1918), this condition mimics the effects of total darkness while allowing the observer to monitor the sexual behavior of the flies. In these tests, mature *D. rajasekari* males were paired with *D. rajasekari* "sex objects" (mature males or females) and their behaviors in "darkness" were compared to behaviors performed by males under normal room lights. Additionally, *D. rajasekari* "sex objects" were tested for their ability to elicit courtship from mature (3 to 5 days old) *D. melanogaster* males in the light and the dark.

RESULTS

Preliminary analysis of the cuticular hydrocarbons from sexually mature *D. rajasekari* males and females indicated that there was little sexual dimorphism in their cuticular hydrocarbons (Table 1). More detailed analyses revealed that the major cuticular component and the only alkadiene from both males and females was a 27-carbon alkadiene, which was identified as 7,11-heptacosadiene (HCD) by interpretation of the mass spectra of its dimethyl disulfide addition products (Figure 1). When the double bonds are separated by one, two, or three methylene groups, the tetrathiomethyl addition results in four-, five-, or six-membered cyclic thioesters substituted with two alkyl chains each containing a methylthio group alpha to the ring (Vincenti et al., 1987). Fragmentation of the dialkyl cyclic thioester between carbons 7 and 8 resulted in mass peaks at 145 and 357 *m/e*. Loss of CH₃-SH from the 357 ion resulted in a peak at 309 *m/e*. Likewise, fragmentation between carbons 11 and 12 resulted in mass peaks at 271 and 231 *m/e*. Subsequent loss of CH₃-SH from the 231 ion resulted in a peak at 183 *m/e*. The other peaks were also consistent with mass spectra of a

TABLE 1. CUTICULAR HYDROCARBONS FROM SEXUALLY MATURE *D. rajasekari* MALES AND FEMALES

ECL	Hydrocarbon	Amount (ng/fly) ^a	
		Females	Males
22.8	9-,7-,5-Tricosenes	6 ± 6	4 ± 1
23.0	<i>n</i> -Tricosane	16 ± 8	13 ± 5
23.6	2-Methyltricosane	2 ± 1	1 ± 1
24.0	<i>n</i> -Tetracosane	3 ± 1	1 ± 1
24.6	2-Methyltetracosane	96 ± 10	74 ± 25
24.7	13-Methyltetracosane	tr	tr
24.8	11-,9-,7-,5-Pentacosenes	10 ± 2	6 ± 1
25.0	<i>n</i> -Pentacosane	44 ± 5	39 ± 14
25.8	8-,6-Hexacosenes	18 ± 1	12 ± 3
26.5	7,11-Heptacosadiene	569 ± 67	335 ± 145
26.6	2-Methylhexacosane	5 ± 3	5 ± 2
26.8	11-,9-,7-Heptacosenes	61 ± 11	43 ± 9
27.0	<i>n</i> -Heptacosane	5 ± 2	4 ± 2
28.6	2-Methyloctacosane	15 ± 5	11 ± 4
Total		850	548

^aMean ± SD. *N* = 6 for each sex. tr = trace quantities.

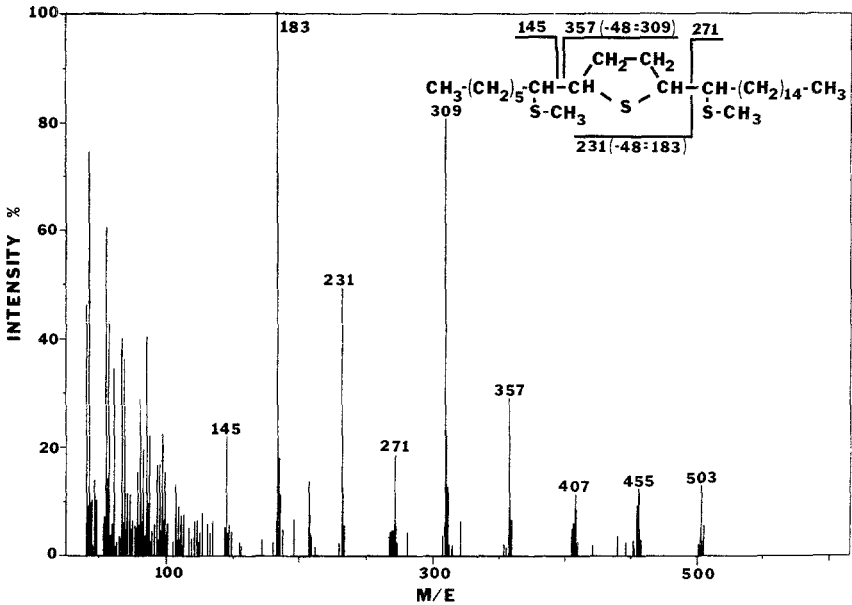


FIG. 1. Mass spectra of the dimethyldisulfide addition product of the alkadiene from *Drosophila rajasekari*.

tetrathiomethyl addition product of a 27-carbon alkadiene. The double bonds were determined to be *Z,Z* by coelution with synthetic (*Z,Z*)-7,11-heptacosadiene.

Alkadienes elute from the AgNO_3 -impregnated silica HPLC column in the order (*E,E*)-, (*E,Z*)-, (*Z,E*)-, and (*Z,Z*)-alkadienes (Bartelt et al., 1986b, and unpublished data), and the 7,11-heptacosadiene from *D. rajasekari* eluted with (*Z,Z*)-alkadienes. The 7,11-heptacosadiene also eluted from both the DB-1 and DB-225 GC columns with the same retention time as (*Z,Z*)-7,11-heptacosadiene from *D. melanogaster*, establishing the *D. rajasekari* HCD as (*Z,Z*)-7,11-heptacosadiene.

Sexually mature *D. rajasekari* males performed an elaborate series of behaviors during courtship (Figure 2). These behaviors included orientation, in which the male positioned himself to face the female; tapping, in which the male contacted the female with his foretarsi; following, in which the male chased the female when she moved away; vibration of the abdomen, in which the male's abdomen vibrated up and down while the male oriented toward the female; wing vibration, in which the male extended one wing and vibrated it (presumably to produce a courtship song); wing extension display, in which the male moved in front of the female, faced her, extended both wings, and made a "scooping"

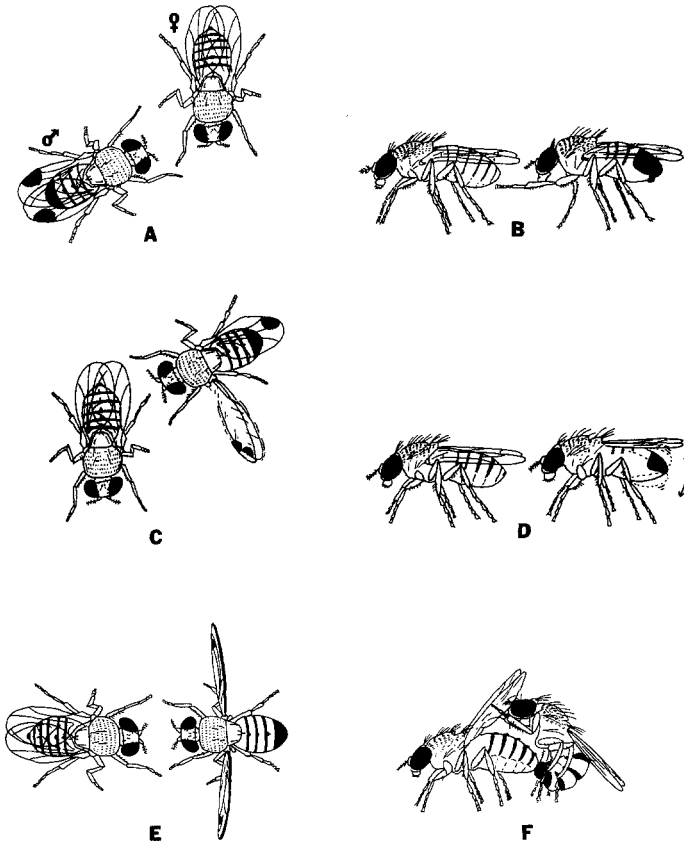


FIG. 2. Courtship behaviors of *Drosophila rajasekari* males: (A) orientation; (B) tapping; (C) extension and vibration of one wing; (D) vibration of the abdomen; (E) wing extension display; (F) attempted copulation.

forward motion with both wings; and attempted copulation, in which the male curled his abdomen under himself and attempted to mount the female.

These behaviors are similar to those performed by courting *D. melanogaster* males, which also orient, tap, follow, extend and vibrate one wing (to produce a courtship song), and attempt copulation. Additionally, *D. melanogaster* males perform a behavior known as licking, where they attempt to contact the courted fly with their proboscis. *D. melanogaster* males do not, however, perform the wing extension display, nor do they vibrate their abdomens as vigorously as do *D. rajasekari* males.

The ontogeny of *D. rajasekari* courtship is summarized in Table 2. Only

TABLE 2. ONTOGENY OF *D. rajasekari* COURTSHIP^a

Age of flies	Mean CI	Number of males performing each behavior
6 hr	3 ± 3	7 None, 2 OTF, 1 OTFWeVAc
12 hr	54 ± 9	1 None, 6 OTFWeV, 3 OTFWeVAc
1 day	60 ± 9	4 OTFWeV, 6 OTFWeVAc
2 days	70 ± 7	3 OTFWeV, 7 OTFWeVAc
3 days	82 ± 5	2 OTFWeV, 7 OTFWeVAc, 1 OTFWeVAc COP
4 days	81 ± 5	1 OTFWeV, 9 OTFWeVAc
5 days	73 ± 8	2 OTFWeV, 8 OTFWeVAc
6 days	79 ± 3	9 OTFWeVAc, 1 OTFWeVAc COP

^aCI = mean ± SE. *N* = 10 for all tests. Behaviors recorded were O, orienting; T, tapping; F, following; We, wing extension; V, abdomen vibration; Ac, attempted copulation; COP, copulation. None = no courtship.

three of the 6-hr-old males performed any of the courtship behaviors, and their CIs were very low. At 12 hr, 1 day and 2 days old, the CIs of the males were much higher, and the majority of the males performed all of the behaviors. At 3 days old, the CIs were at their highest level, the majority of the males performed all of the behaviors, and one male successfully copulated within the observation period. Males that were 4 to 6 days old were not significantly different from 3-day-old males in terms of CIs or behaviors performed. Therefore, it was concluded that males become sexually mature (as defined in Methods and Materials) at the age of 3 days.

Drosophila rajasekari females that were 3 to 5 hr, 1 day, and 3 to 5 days old were all sexually attractive to mature *D. rajasekari* males. In addition, 3- to 5-hr-, 1-day-, and 3- to 5-day-old *D. rajasekari* males also elicited courtship from mature *D. rajasekari* males, although the 1-day- and 3- to 5-day-old males were significantly less attractive than the 3- to 5-hr-old males ($P < 0.001$; Mann-Whitney test). These results are summarized in Table 3.

Since mature *D. rajasekari* males performed courtship toward 3- to 5-day-old *D. rajasekari* females and males in white light, they were then tested for their ability to perform courtship toward those flies in the dark (Table 4). Only one *D. rajasekari* male courted in the dark. This male oriented, tapped, and extended his wing in response to a *D. rajasekari* female.

Table 4 also shows the results of courtship tests involving *D. melanogaster* and *D. rajasekari*. As expected, *D. melanogaster* males performed vigorous amounts of courtship in response to *D. rajasekari* males and females in both the light and dark experimental conditions. Mature *D. rajasekari* males did not, however, actively court *D. melanogaster* females.

TABLE 3. COURTSHIP OF SEXUALLY MATURE *D. rajasekari* MALES IN RESPONSE TO DIFFERENT "SEX OBJECTS"^a

Sex object	Mean CI of <i>D. rajasekari</i> males
3- to 5-hr-old <i>D. rajasekari</i> female	76 ± 3
1-day-old <i>D. rajasekari</i> female	68 ± 10
3- to 5-day-old <i>D. rajasekari</i> female	82 ± 5
3- to 5-hr-old <i>D. rajasekari</i> male	67 ± 4
1-day-old <i>D. rajasekari</i> male	22 ± 8
3- to 5-day-old <i>D. rajasekari</i> male	17 ± 8

^aCI = mean ± SE. *N* = 10 for all tests. The *D. rajasekari* males whose courtship was recorded were 3 to 5 days old. In tests in which two males were tested together, one of the males had the tip of one wing removed. In half the tests the courting male's wing was clipped; in the other half, the sex object's wing was clipped.

TABLE 4. COURTSHIP IN THE LIGHT AND IN THE DARK

Courting male	Sex object	Illumination	CI
<i>D. rajasekari</i>	<i>D. rajasekari</i> female	light	82 ± 5
<i>D. rajasekari</i>	<i>D. rajasekari</i> female	dark	2 ± 2
<i>D. rajasekari</i>	<i>D. rajasekari</i> male	light	17 ± 8
<i>D. rajasekari</i>	<i>D. rajasekari</i> male	dark	0 ± 0
<i>D. rajasekari</i>	<i>D. melanogaster</i> female	light	1 ± 0
<i>D. melanogaster</i>	<i>D. rajasekari</i> female	light	78 ± 6
<i>D. melanogaster</i>	<i>D. rajasekari</i> female	dark	60 ± 8
<i>D. melanogaster</i>	<i>D. rajasekari</i> male	light	90 ± 3
<i>D. melanogaster</i>	<i>D. rajasekari</i> male	dark	40 ± 3

^aCI = mean ± SE. *N* = 10 for all tests. Dark = illuminated by red light. Light = illuminated by white incandescent light. All flies were 3 to 5 days old. In tests in which two males were tested together, one of the males had the tip of one wing surgically removed. In half the tests the courting male's wing was clipped, in the other half, the sex object's wing was clipped.

DISCUSSION

We have shown that little sexual dimorphism exists with respect to the cuticular hydrocarbons extracted from *D. rajasekari* males and females. In addition, a detailed analysis revealed the major cuticular component of these flies to be the 27-carbon alkadiene, (*Z,Z*)-7,11-heptacosadiene (HCD).

Our behavioral assays have shown that *D. rajasekari* males perform a complex series of courtship behaviors prior to mating and that these males are sexually attracted to other *D. rajasekari* males as well as *D. rajasekari* females. Additionally, *D. rajasekari* males and females are attractive both when they are young and when they are mature. This situation is similar to that found in *D. melanogaster* in that virgin females are attractive throughout their lives, but different in that *D. melanogaster* males, which are attractive when immature, lose their sex appeal as they mature (Jallon and Hotta, 1979; McRobert and Tompkins, 1983; Curcillo and Tompkins, 1987). The sex appeal of *D. rajasekari* males declines as they age, but mature males still elicit relatively high levels of courtship from other males.

D. rajasekari females and males were also sexually attractive to *D. melanogaster* males. Furthermore, since *D. melanogaster* males performed vigorous courtship displays toward *D. rajasekari* females and males in the absence of light, the cues responsible for the initiation of this cross-species courtship behavior are probably olfactory. Of course, this is not unexpected, since mature *D. rajasekari* produce large quantities of HCD, a compound known to be a major courtship-stimulating pheromone in *D. melanogaster* (Antony and Jallon, 1982; Antony et al., 1985).

On the other hand, *D. rajasekari* males performed almost no courtship in response to *D. melanogaster* females, which are known to produce large quantities of HCD. These results suggest either that *D. rajasekari* males are not stimulated to court by HCD or that *D. melanogaster* females produce some compound that inhibits the courtship of *D. rajasekari* males. If the first of these possibilities is true, then the sex appeal of *D. rajasekari* females and males to mature *D. rajasekari* males must be caused by some factor other than HCD. This stimulus could be visual, olfactory, or both. The fact that one *D. rajasekari* male courted a *D. rajasekari* female in the dark indicates that there may be some olfactory cue involved, but it is unlikely that this cue is HCD.

In summary, our results further substantiate the fact that (Z,Z)-7,11-heptacosadiene (HCD) stimulates courtship from mature *D. melanogaster* males, even when this courtship-stimulating cue is provided by flies that are not *D. melanogaster* females. Furthermore, *D. rajasekari* represents a species in which males and females produce large quantities of HCD, but do not apparently use it as a courtship-stimulating pheromone.

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TERPENE ALCOHOL PHEROMONE PRODUCTION BY
Dendroctonus ponderosae AND *Ips paraconfusus*
(COLEOPTERA: SCOLYTIDAE) IN THE
ABSENCE OF READILY CULTURABLE
MICROORGANISMS¹

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Abstract—*Dendroctonus ponderosae* Hopkins and *Ips paraconfusus* Lanier of both sexes produced most of their complement of terpene alcohols at normal to elevated levels in the absence of readily culturable microorganisms. However, there was some evidence that microbial involvement may be required by male *I. paraconfusus* to produce ipsenol and ipsdienol at normal levels. Increased levels of certain terpene alcohols found in axenically reared or streptomycin-fed beetles suggest that symbiotic microorganisms may be responsible for breaking down pheromones and other terpene alcohols. There was also evidence for microbial involvement in the production of the antiaggregation pheromone verbenone in *D. ponderosae*. This compound was not produced in quantifiable levels by axenically reared or streptomycin-fed beetles exposed to α -pinene as vapors or through feeding, but was found in wild *D. ponderosae* exposed to α -pinene through feeding on bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann.

Key Words—*Dendroctonus ponderosae*, *Ips paraconfusus*, Coleoptera, Scolytidae, pheromones, terpene alcohols, axenic-rearing, bark beetles, microorganisms.

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INTRODUCTION

The role of symbiotic microorganisms in pheromone production by bark beetles has previously been studied by isolating and culturing these microbes and examining their capacities to produce terpene alcohol pheromones *in vitro*. Certain species of microorganisms found in association with scolytids produced some of their hosts' terpene alcohol pheromones when exposed to monoterpene precursors of those pheromones (Brand et al., 1975; Chararas et al., 1980). This finding is not surprising since these microorganisms are exposed in nature to the same monoterpenes as the host beetles, and many species of microorganisms can oxidize monoterpene hydrocarbons (Bhattacharyya et al., 1960; Prema and Bhattacharyya, 1962; Shukla et al., 1968; Fonken and Johnson, 1972; Keislich, 1976). However, there is little evidence that microbe-produced terpene alcohols are being used in nature as pheromones by the host beetles or that these compounds are even being produced by the symbionts in quantities that are biologically significant to the host beetles.

In order to assess the importance of the microbial contribution to overall pheromone production, it is essential to ascertain the metabolic capabilities of beetles that are relatively free of microbial symbionts. Chararas (1980) claimed that after *Ips sexdentatus* Boerner, *Ips typographus* (L.), and *Ips acuminatus* Gyllenhal had been fed a wide-spectrum antibiotic, their frass showed slightly reduced attractiveness to other beetles of the same species. Chararas also reported that the frass of antibiotic-fed beetles had more of the monoterpenes α -pinene, β -pinene, and Δ^3 -carene, and less of the conversion products of these compounds, than did frass from normal beetles. Byers and Wood (1981), found that *Ips paraconfusus* (Lanier) that were fed streptomycin were unable to convert myrcene to the pheromones ipsenol and ipsdienol, while the synthesis of *cis*-verbenol from α -pinene was not inhibited. They interpreted these results to indicate that symbiotic bacteria sensitive to streptomycin are involved in the synthesis of ipsenol and ipsdienol and that *cis*-verbenol is either synthesized by insect cells or by microorganisms that are unaffected by streptomycin at the concentrations used. However, while these results suggest that microorganisms are involved in pheromone production by *Ips* species, treatment of insects with dietary antibiotics can produce adverse physiological effects in the insects (Greenberg, 1970). It is possible that reductions in pheromone production through streptomycin-feeding (Chararas, 1980; Byers and Wood, 1981) were the result of such effects.

Axenic rearing is a useful technique for the study of symbiosis in insects (Rodriguez, 1966; Vanderzant, 1974). The development of a technique for rearing bark beetles axenically (Bedard, 1966; Whitney and Spanier, 1982) has

provided an opportunity to study the production of pheromones by bark beetles with reduced levels of microorganisms without the use of antibiotics.

The mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the California five-spined ips, *I. paraconfusus*, were chosen for this study because their pheromone chemistry is fairly well understood and because much of the research that has indicated that microorganisms are involved in the production of bark beetle pheromones has been conducted on *I. paraconfusus* (Brand et al., 1975; Byers and Wood, 1981), and *Dendroctonus* species (Brand et al., 1976, 1977; Brand and Barras, 1977).

D. ponderosae infests living *Pinus* species throughout western North America (Wood, 1982). Females convert the host tree monoterpene α -pinene to the aggregation pheromone *trans*-verbenol (Hughes, 1973; Pitman, 1971) and a variety of other terpene alcohols such as myrtenol and *cis*-verbenol. Two bicyclic ketals, *exo*-brevicommin and frontalin, which are of uncertain biosynthetic origin (Vanderwel and Oehlschlager, 1987), are apparently concentration-dependent, multifunctional pheromones, acting to promote aggregation (Rudinsky et al., 1974; Conn et al., 1983; Borden et al., 1983, 1987) or antiaggregation (Rudinsky et al., 1974; Pitman et al., 1978; Ryker and Rudinsky, 1982; Ryker and Libbey, 1982; Borden et al., 1987). *endo*-Brevicommin (Ryker and Rudinsky, 1982), verbenone (Ryker and Yandell, 1983; Borden et al., 1987), pinocarvone (Libbey et al., 1985), and ipsdienol (Hunt and Borden, 1988) all have activity as antiaggregation pheromones, while the monoterpenes α -pinene (Pitman, 1971), myrcene, and terpinolene (Billings et al., 1976; Conn et al., 1983; Borden et al., 1983) act as synergistic kairomones in promoting aggregation.

I. paraconfusus is usually found in weakened or fallen *Pinus* species in Oregon, Nevada, and California (S.L. Wood, 1982). It also infests the upper boles of trees killed by the western pine beetle, *Dendroctonus brevicomis* LeConte and in some cases kills the upper crown of mature ponderosa pines, *Pinus ponderosa* Laws or entire young trees (Struble and Hall, 1955). *I. paraconfusus* converts α -pinene to *cis*-verbenol (Renwick et al., 1976) and myrcene to ipsdienol (Byers et al., 1979; Hendry et al., 1980) and then ipsenol (Fish et al., 1979). It utilizes ipsenol, ipsdienol, and *cis*-verbenol as aggregation pheromones (Wood et al., 1968).

Conn et al. (1984) established that axenically reared *D. ponderosae* and *I. paraconfusus* are capable of producing certain terpene alcohol pheromones. However, their results were somewhat inconsistent, and they did not establish whether axenically reared insects are capable of producing these compounds at levels equal to normal insects with their full complement of microorganisms. Our objective was to assess in as comprehensive and definitive a manner as

possible the effects of axenic rearing and antibiotic feeding on the production of terpene alcohols and ketones from α -pinene and myrcene in both sexes of *D. ponderosae* and *I. paraconfusus*.

METHODS AND MATERIALS

Collection of Insects and Host Material. Lodgepole pines, *P. contorta* var. *latifolia*, infested with *D. ponderosae* and uninfested lodgepole and ponderosa pine, *Pinus ponderosa* Laws., were collected near Princeton, British Columbia. Adult *I. paraconfusus* were obtained from D.L. Wood (Department of Entomological Sciences, University of California-Berkeley, California 94720), and a colony was maintained on ponderosa pine bolts. Adult *D. ponderosae* and *I. paraconfusus* were collected daily after they emerged from caged logs kept at 27°C. Emerged beetles were stored on moistened paper towels at 2–4°C in loosely capped, screw-top jars. When beetles were needed for experiments, jars that had been stored a maximum of two weeks were rewarmed to room temperature, and normal and healthy-appearing individuals were selected.

Axenic Rearing. An axenic rearing technique (Whitney and Spanier, 1982) was used to obtain beetles that were devoid of readily culturable microorganisms. The outer bark was shaved off fresh pine bolts (lodgepole pine for *D. ponderosae* and ponderosa pine for *I. paraconfusus*), and the exposed phloem was removed and cut into 1-cm² pieces. The phloem was frozen at –25°C, ground in a prechilled Waring blender, and passed through No. 12 mesh screen. Grinding and sieving were conducted in a –25°C walk-in cold-room, and the ground phloem was stored in plastic bags at –20°C. When diet was required for insect rearing, the phloem was thawed, and water and dehydrated brewer's yeast were added at 50% and 10%, respectively, of the weight of the phloem. The mixed diet was dispensed into 19 × 55-mm shell vials at approximately 0.75 g/vial using a 10-ml plastic syringe with the bottom removed. The vials were capped with Morton stainless-steel culture closures (18 mm), pasteurized in an Amsco isothermal sterilizer for 1.0 hr at 85°C, held at room temperature for four days, and then repasteurized. This double treatment of the vials in the isothermal sterilizer yielded sterile diet.

Adult female *D. ponderosae* or male *I. paraconfusus* were individually caged onto fresh bolts of lodgepole or ponderosa pine, respectively, using gelatin capsules (Lanier and Wood, 1968). After 24 hr, a mate was introduced to each capsule and the bolts were kept at 26–28°C. After 10 days for *D. ponderosae* or seven days for *I. paraconfusus*, the bark was removed. Using a blunt probe, the eggs were collected from the parent galleries, transferred to moistened filter paper in a Petri dish, sealed in a plastic bag to prevent moisture loss,

and incubated at 22–24°C in the dark. Incubating eggs were examined twice daily for *D. ponderosae* and three times daily for *I. paraconfusus*. Fourth-stage eggs (Reid and Gates, 1970) were surface-sterilized in 0.1% mercuric chloride (Fisher Scientific Co., Fair Lawn, New Jersey), rinsed six times in sterile distilled water, and placed individually in the vials of sterilized diet. Sterilized Pasteur pipets were used to transfer the eggs and the procedure was performed aseptically on a sterile air bench. The eggs were allowed to mature to adults in the vials, and emergent, axenically reared adults remained in the rearing vials until used in experiments.

Checks for aerobic microorganisms in the sterilized diet, as well as externally and internally from all stages of the developing axenically reared insects, were done on malt extract agar, potato dextrose agar, and plate count agar. Axenically reared adult beetles were also examined for microorganisms on their exposed cuticle using scanning electron microscopy.

Antibiotic Feeding. An antibiotic feeding technique similar to that developed by Byers and Wood (1981) was used to obtain beetles with reduced levels of symbiotic microorganisms. A mixture of 35 g of powdered cellulose (alpha-cellulose, Sigma Chemical Co., St. Louis, Missouri), 8 g of sucrose and 22 g of ground pine phloem (lodgepole and ponderosa pine phloem for *D. ponderosae* and *I. paraconfusus*, respectively) was added to 62 ml of distilled water containing 10 mg of streptomycin sulfate (Sigma) per milliliter of water. The ingredients were thoroughly mixed and then dispensed into 19 × 55-mm shell vials at approximately 0.75 g/vial using a 10-ml plastic syringe with the bottom removed. An adult beetle was added to each vial and the insects were allowed to feed for 96 hr at 22–24°C. Petri dishes were not used as in Byers and Wood (1981), as the use of individual vials made it possible to eliminate from the experiment beetles which did not feed.

Maturation Experiments. The term “wild” in this paper indicates that the beetles have not had their normal complement of microorganisms removed or reduced. When wild adults of a known age were required, the bark was removed from infested bolts and pupae were collected, placed in Petri dishes containing moistened filter paper, and examined daily until eclosion (the term “eclosion” in this paper refers specifically to molt from pupa to adult; posteclosion age refers to the number of days as an adult). Callow adults were removed from the Petri dishes and allowed to mature in individual vials until reaching the desired age (we use “mature” to designate beetles of the posteclosion age at which peak levels of terpene alcohol production were observed to occur). Since fungi found in the pupal chamber are thought to be important in the maturation feeding of callow scolytids (Whitney, 1971; Barras, 1973), moistened bark from around the pupal chambers in the infested bolt was included in each vial.

Vials containing axenically reared individuals were examined daily near

the time of eclosion so that the maturity of adult beetles used in experiments was known.

Pheromone Production and Extraction Techniques. Pheromone production was induced using one of two methods: (1) Beetles that had bored in the inner bark of pine logs for 24 hr were chipped out and held over Dry Ice until a sufficient number had accumulated for dissection. (2) Beetles were placed individually in upright, open-ended glass cylinders (1.4 cm ID) (Conn, 1981) inside a 500-ml glass jar. Open vials (1.8 ml screw-cap) containing 25 μ l of the pheromone precursors α -pinene and/or myrcene were also placed in the jar, and the jar was sealed and held in the dark for 24 hr.

Beetle abdomens were removed using fine forceps, and immediately immersed in 100 μ l of double-distilled pentane in individual 1.8-ml glass vials over Dry Ice. The excised abdomens included the hindgut, Malpighian tubules, and a large portion of the midgut. Each abdomen was macerated with the tip of a spatula in the pentane over Dry Ice, and the vial was then sealed and allowed to sit at room temperature for approximately 15 min. The pentane extract was then transferred with a syringe into a clean vial. The macerated tissue was rinsed twice each with 25 μ l of double distilled pentane at room temperature, and this was added to the clean vial, which was then closed with a Teflon-lined screw-cap, and stored at -20°C .

Gas Chromatographic Analyses. Single beetle extracts were analyzed on a Hewlett Packard 5880A gas chromatograph equipped with a capillary inlet system and a flame ionization detector. A glass capillary column (30 m \times 0.66 mm ID) coated with SP-1000 (Supelco, Inc., Bellefonte, Pennsylvania) was used with the temperature set at 120°C for 2 min, and then increasing by $4^{\circ}\text{C}/\text{min}$ to 180°C . The injection port temperature was 260°C , the flame ionization detector temperature was 275°C , and helium was used as the carrier gas. A known quantity of 3-octanol was added to the distilled pentane used to extract the abdomens, and the same quantity of 2-octanol was added following extraction. The area under the 3-octanol peak was used as a reference for calculating the quantities of each compound present in each sample, and the ratio of 3- to 2-octanol recovered was used to monitor the loss of volatile compounds during the extraction process.

Each day that samples were analyzed by gas chromatography, a standard sample made up of 2- and 3-octanol, α -pinene, myrcene, *cis*- and *trans*-verbenol, myrtenol, verbenone, ipsenol, ipsdienol, and myrcenol was also analyzed for comparison of retention times. In addition, these compounds were periodically added to an abdominal extract, and that mixture was analyzed to ensure correct identification by cochromatography with unknown compounds in the extract. Selected extracts were analyzed using gas chromatography-mass spectroscopy to ensure proper identification of compounds. For most samples, the detection limit for compounds of interest was set at 5 ng/abdomen.

Experiments Conducted. Twenty-one experiments were conducted with *D. ponderosae* and *I. paraconfusus* adults of both sexes, with experiment number and objectives as outlined below. Details of experimental treatments and replicates are given in Tables 1-3, and Figures 1-3.

Female D. ponderosae.

- I. To assess the effect of axenic rearing or streptomycin feeding on the conversion of α -pinene into terpene alcohols.
- II. To assess the effect of maturation on the ability of axenically reared beetles to produce terpene alcohols.
- III. To compare the effect of various degrees of maturation on the ability of axenically reared and wild beetles to produce terpene alcohols.
- IV. To compare the levels of terpene alcohol production by mature, axenically reared beetles, mature, wild beetles, and emerged, wild beetles.
- V and VI. To compare the effects of streptomycin-feeding on terpene alcohol production in beetles exposed to α -pinene either as vapors or through feeding.
- VII. To determine the effects of axenic rearing on the conversion of ingested α -pinene into terpene alcohols.
- VIII. To determine the ability of females to convert myrcene into terpene alcohols.

Male D. ponderosae.

- IX and X. To determine the effects of axenic rearing and streptomycin feeding on the conversion of α -pinene (experiment IX) and myrcene (experiment X) vapors into terpene alcohols.

Male I. paraconfusus.

- XI and XII. To compare the effect of various degrees of maturation on the ability of axenically reared and wild beetles to produce α -pinene-derived (experiment XI) and myrcene-derived (experiment XII) terpene alcohols.
- XIII. To compare the levels of myrcene-derived terpene alcohol production by mature, axenically reared beetles; mature, wild beetles; and emerged, wild beetles.
- XIV. To compare the levels of terpene alcohol production by mature, axenically reared beetles; mature, wild beetles; and emerged, wild beetles.
- XV. To assess the effects of streptomycin-feeding on terpene alcohol production in wild beetles exposed to α -pinene vapors.

- XVI and XVIII. To determine the effects of streptomycin feeding (experiment XVI) or axenic rearing (experiment XVIII) on terpene alcohol production in beetles exposed to α -pinene through feeding.
- XVII. To compare the efficacy of two different batches of streptomycin in reducing the production of myrcene-derived terpene alcohols.
- XIX. To compare the levels of terpene alcohol production in beetles exposed to myrcene vapors, myrcene and α -pinene vapors simultaneously, or the myrcene and α -pinene encountered while feeding on bolts of *P. ponderosa*.
- XX. To determine the effects of distending the guts of *I. paraconfusus* with powdered cellulose on the conversion of myrcene vapors to ipsdienol and ipsenol.

Female I. paraconfusus.

- XXI. To determine the effects of axenic rearing and streptomycin feeding on the conversion of α -pinene vapors into terpene alcohols.

Statistical Analysis. The pheromone data were tested for homogeneity of variances using Cochran's *C* test as well as the Bartlett-Box *F* test using SPSS^x (1983). The data were heteroscedastic; therefore, they were analyzed using the Kruskal-Wallis test (Sokal and Rohlf, 1981) followed by a nonparametric multiple comparisons test (Conover, 1980, p. 231), $P < 0.05$.

RESULTS AND DISCUSSION

Axenically Reared Beetles. During axenic rearing, the few vials with visible signs of contamination were discarded. In one batch of axenically reared *D. ponderosae*, there were many contaminated vials, and closer examination revealed numerous mites in the vials. These mites, which are frequently associated with wild *D. ponderosae*, are small enough that they can enter the shell vials after capping with the steel culture closures. Contamination with mites, and the fungi that they carry, was subsequently avoided by ensuring that the capped vials were not handled or stored in areas of the laboratory where wild beetles were handled.

Checks for aerobic microorganisms in the vials of sterilized diet, as well as externally and internally from all stages of axenically reared *D. ponderosae* and *I. paraconfusus*, revealed no microorganisms. In developing the technique, Whitney and Spanier (1982) had conducted even more extensive culturing on axenically reared *D. ponderosae*, which also revealed no microorganisms. In addition, no microorganisms other than brewer's yeast cells were observed on

the external cuticular surface of axenically reared adults examined by scanning electron microscopy.

Although all of the above checks and precautions indicate that beetles obtained using the axenic rearing technique are likely to be truly axenic, it is possible that transovarially transmitted, obligate symbionts may still be associated with these beetles. Therefore, we refer to beetles obtained using this technique as "axenically reared" or "microbe reduced," and not "axenic."

Of the *D. ponderosae* eggs that were surface sterilized and placed in vials of sterilized diet, generally 60–80% survived to adulthood. Only 40–60% of axenically reared *I. paraconfusus* survived, probably because the eggs of this species are more fragile and were more easily damaged during handling.

Initial Experiments on Pheromone Production by Axenically Reared Beetles. Experiment 1 (Table 1) failed to verify the results of Conn et al. (1984) in which female *D. ponderosae* free of readily culturable microorganisms contained far more *trans*-verbenol than wild control beetles. Rather, axenically reared beetles in experiment 1 contained *trans*- and *cis*-verbenol and myrtenol at levels too low to be quantified (Table 1, experiment I). Streptomycin-fed beetles and wild controls also contained unusually low levels of these terpene alcohols (Table 1, experiment I).

In a subsequent experiment, beetles from the same batch of axenically reared, female *D. ponderosae*, which were allowed to mature for a further 14 days, produced significant levels of *trans*- and *cis*-verbenol, and myrtenol when exposed to α -pinene vapors (Table 1, experiment II). Normal levels of production were found in wild, control beetles. The quantities of *trans*- and *cis*-verbenol produced by axenically reared beetles in this experiment were not significantly different from those produced by wild emerged beetles, although the production of myrtenol was significantly different in the axenically-reared individuals (Table 1, experiment II).

Effect of Maturation on Terpene Alcohol Production in Female D. ponderosae. The axenically reared female *D. ponderosae* in experiment I were fully darkened adults, and it was initially assumed that they had reached physiological maturity. However, since individuals taken from the same batch 14 days later produced terpene alcohols at higher levels (Table 1, experiment II), and because no record was made of the age of the axenically reared females used by Conn et al. (1984), we hypothesized that maturation beyond the point of darkening of axenically reared *D. ponderosae* was required before peak terpene alcohol production occurred.

To test this hypothesis, we exposed axenically reared females of varying posteclosion ages to α -pinene vapors. Axenically reared individuals of 5 and 15 days posteclosion produced *trans*- and *cis*-verbenol and myrtenol at relatively low levels, while levels in beetles of 25 and 35 days posteclosion were much higher than those in wild emerged beetles (Figures 1–3). Thus a three- to four-week maturation period is apparently required before adult *D. ponderosae*

TABLE 1. OXYGENATED MONOTERPENE PRODUCTION IN AXENICALLY REARED, STREPTOMYCIN-FED, AND WILD *Dendroctonus ponderosae* FEMALES (EXPERIMENTS I-VIII) AND MALES (EXPERIMENTS IX AND X)

Exp. No.	Sex	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle) ^a			
				<i>trans</i> -verbenol	<i>cis</i> -verbenol	myrtenol	
I	Females	Wild/air	7	<5 a	<5 a	<5 a	
		Wild/ α -pinene vapors	8	46 b	20 a	7 a	
		Wild/streptomycin/air	7	20 ab	8 a	<5 a	
		Wild/streptomycin/ α -pinene vapors	8	44 b	17 a	7 a	
		Axenically reared/air	8	<5 a	<5 a	<5 a	
		Axenically reared/ α -pinene vapors	7	<5 a	<5 a	<5 a	
		Axenically reared/streptomycin/air	8	8 a	<5 a	<5 a	
II	Females	Axenically reared/streptomycin/ α -pinene vapors	7	<5 a	<5 a	<5 a	
		Wild/air	10	<5 a	<i>cis</i> -verbenol	myrtenol	
		Wild/ α -pinene vapors	8	446 b	70 b	79 b	
		Axenically reared/air	9	7 a	<5 a	<5 a	
		Axenically reared/ α -pinene vapors	8	177 b	26 b	28 c	
		Wild/emerged/air	10	<5 a	<5 a	<5 a	verbenone
		Wild/emerged/ α -pinene vapors	11	194 b	37 b	37 b	<5 a
IV	Females	Wild/mature/air	6	<5 a	<5 a	<5 a	
		Wild/mature/ α -pinene vapors	6	637 bc	49 bc	127 c	
		Axenically reared/mature/air	10	<5 a	<5 a	<5 a	
		Axenically reared/mature/ α -pinene vapors	10	1216 c	87 c	112 c	
		Wild/air	7	<5 a	<i>cis</i> -verbenol	myrtenol	
		Wild/ α -pinene vapors	7	730 b	133 b	252 b	
		Wild/streptomycin/air	7	<5 a	<5 a	<5 a	
V	Females	Wild/streptomycin/ α -pinene vapors	7	2056 b	104 b	172 b	

VI	Females	Wild/air Wild/ α -pinene vapors Wild/ <i>P. contorta</i> Wild/streptomycin/air Wild/streptomycin/ α -pinene vapors Wild/streptomycin/ <i>P. contorta</i>	6 9 6 7 10 5	<i>trans</i> -verbenol <5 a 261 b 17 a <5 a 3375 c 17 a	<i>cis</i> -verbenol <5 a 30 b <5 a <5 a 155 c <5 a	myrtenol <5 a 39 b <5 a <5 a 264 c <5 a
VII	Females	Wild/air Wild/ <i>P. contorta</i> Axenically reared/air Axenically reared/ <i>P. contorta</i>	12 12 11 12	<i>trans</i> -verbenol <3 a 241 b <3 a <3 a	verbenone <3 a 30 b <3 a <3 a	
VIII	Females	Wild/air Wild/myrcene Wild/streptomycin/air Wild/streptomycin/myrcene vapors	7 9 9 8	ipsdienol <10 a <10 a <10 a <10 a	(Z)-1-myrcenol <5 a 11 a <5 a 10 a	(E)-1-myrcenol <5 a 415 b <5 a 641 b
IX	Males	Wild/air Wild/ α -pinene vapors Wild/streptomycin/air Wild/streptomycin/ α -pinene vapors Axenically reared/air Axenically reared/ α -pinene vapors	7 7 7 11 9 10	<i>trans</i> -verbenol <5 a 169 b <5 a 1534 c <5 a 1735 c	<i>cis</i> -verbenol <5 a 98 b <5 a 482 c <5 a 571 c	myrtenol <5 a 91 b <5 a 180 c <5 a 207 c
						unknown (R, 14.74) <5 a 55 b <5 a 103 b
X	Males	Wild/air Wild/myrcene vapors Wild/streptomycin/air Wild/streptomycin/myrcene vapors Axenically reared/air Axenically reared/myrcene vapors	10 11 10 10 10 12	ipsdienol <10 a 1906 b <10 a 1867 b <10 a 3122 b	(Z)-1-myrcenol <5 a 156 b <5 a 57 c <5 a 110 bc	(E)-1-myrcenol <5 a 1341 b <5 a 277 c <5 a 222 c
						unknown (R, 14.74) <5 a 167 b <5 a 36 c <5 a 62 c

^aData analyzed using the Kruskal-Wallis test followed by a nonparametric multiple comparisons test (Conover, 1980; p. 231), $P < 0.05$. Means within a column for each experiment followed by the same letter are not significantly different.

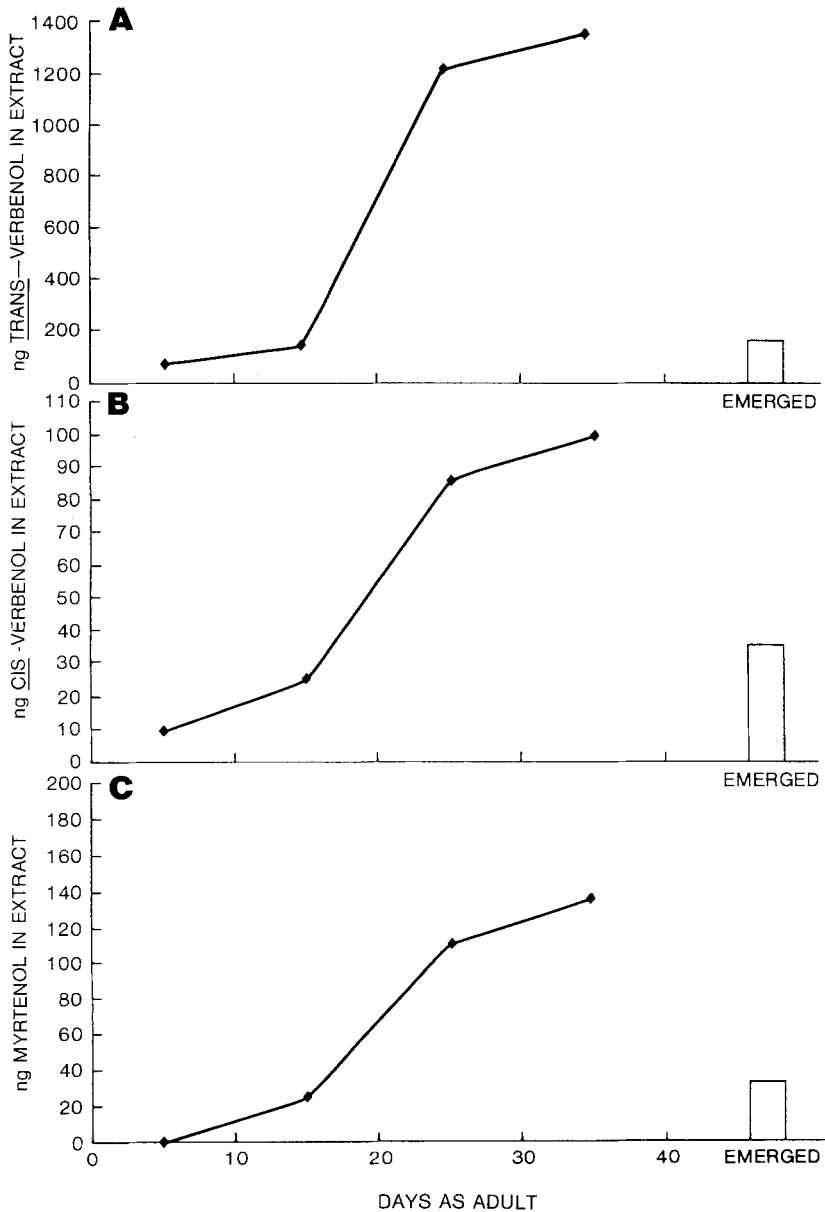


FIG. 1. Quantities (nanograms) of *trans*-verbenol (A, D), *cis*-verbenol (B, E), and myrtenol (C, F), produced in experiment III by individual axenically reared (A-C) and wild (D-F) female *D. ponderosae* of various posteclosion ages that were exposed to α -pinene vapors. Numbers of beetles used for each treatment were as follows. Axenically reared: 5 days, 9; 15 days, 10; 25 days, 10; 35 days, 8. Wild, group 1: 6 days, 6; 16 days, 6; 27 days, 6; 40 days, 9. Wild, group 2: 5 days, 11; 15 days, 8; 25 days, 10; 35 days, 10.

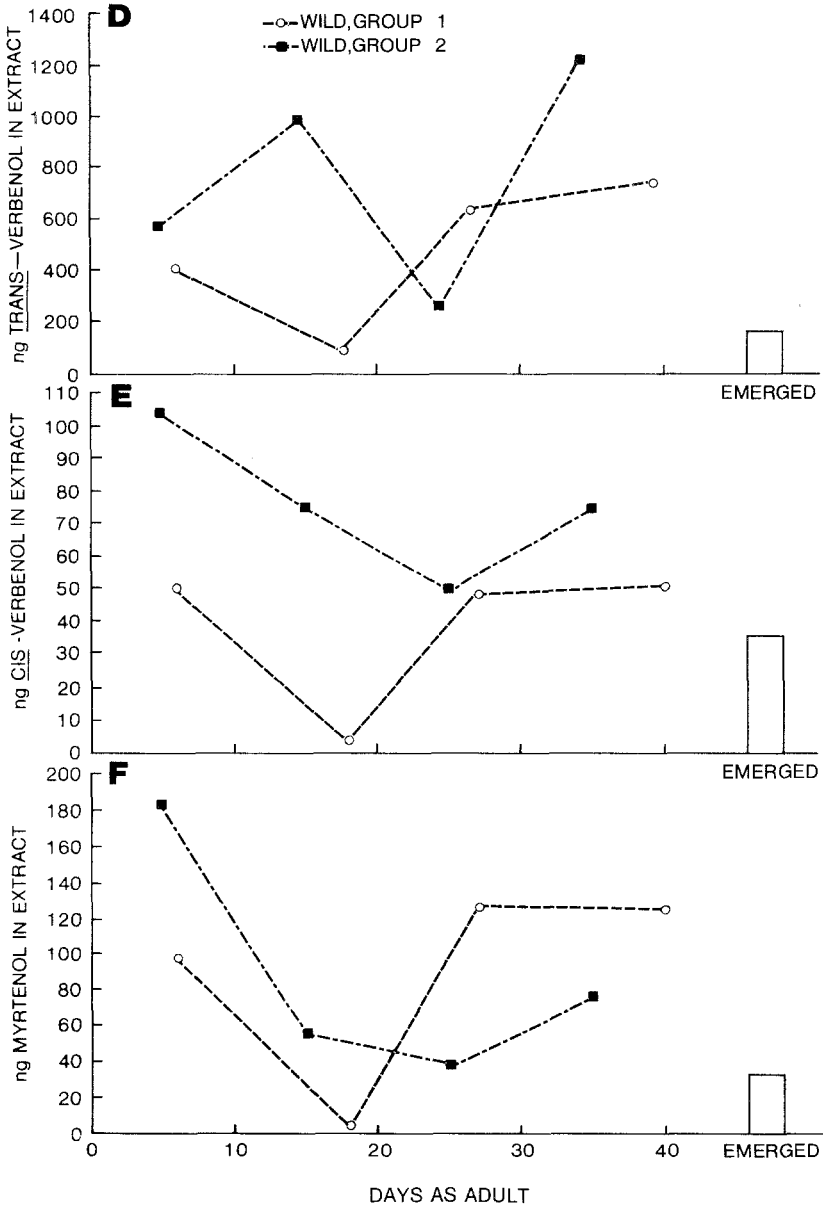


FIG. 1. Continued.

develop maximal enzymatic capacity for the oxidation of α -pinene into terpene alcohols.

Since the posteclosion age of *D. ponderosae* is evidently important in determining the physiological maturity of axenically reared beetles, much of the data reported by Conn et al. (1984) must be questioned. Although Conn et al. (1984) did establish that axenically reared *D. ponderosae* can produce terpene alcohol pheromones, it is likely that the generally low levels of production of these compounds reported by Conn et al. (1984) simply reflect the use of immature beetles. The production of very high levels of *trans*-verbenol by axenically reared female *D. ponderosae* in one experiment (9900 ng/beetle) probably indicates the fortuitous use of mature beetles.

The pattern of terpene alcohol production in relation to age for wild female *D. ponderosae* was different than that for axenically reared individuals (Figure 1). During the first 15 days posteclosion, the production of *trans*- and *cis*-verbenol and myrtenol in two groups of beetles was moderate to high, then declined sharply, and returned to higher levels in older beetles (Figure 1). The terpene alcohol production in wild callow adults of 5–15 days posteclosion is not likely to be due to production by microorganisms since wild beetles of 18–25 days posteclosion, which should have the same complement of symbiotic microorganisms, produced terpene alcohols at much lower levels (Figure 1). Possibly the *cis*- and *trans*-verbenol and myrtenol produced by wild beetles of 5–15 days posteclosion is metabolized from derivatized α -pinene sequestered by the insects as larvae or pupae (Hughes, 1975), rather than by conversion of inhaled α -pinene vapors. Axenically reared callow adults could not produce significant quantities of terpene alcohols from sequestered precursors since the monoterpenes in the ground phloem in the axenic diet are virtually eliminated during the sterilization process (Table 2). The increase in terpene alcohol production in wild beetles 25 days posteclosion would, as in axenically reared beetles, be due to the maturation of the enzyme systems responsible for oxidizing the monoterpenes encountered as the mature adult attacks a new host.

The peak levels of terpene alcohol production were generally much higher

TABLE 2. EFFECT OF PASTEURIZATION ON MONOTERPENE CONTENT OF GROUND PHLOEM IN AXENIC DIET

Phloem source	Reduction %	
	α -Pinene	Myrcene
<i>Pinus contorta</i> var. <i>latifolia</i>	99	98
<i>Pinus ponderosa</i>	78	90

in wild beetles of 27–40 days posteclosion than in wild emerged beetles (Figure 1). This difference apparently indicates that wild emerged beetles are not yet capable of peak pheromone production. There is evidence that certain species of bark beetles undergo a period of obligatory dispersal before they become responsive to olfactory signals associated with their hosts or other beetles. Flight exercise was found to increase positive responses to attractive semiochemicals in *Dendroctonus frontalis* Zimmerman (Andryszak et al., 1982), *Dendroctonus pseudotsugae* Hopkins (Bennett and Borden, 1971), *Trypodendron lineatum* (Olivier) (Graham 1959, 1962; Bennett and Borden, 1971), and *Scolytus multistriatus* (Marsham) (Choudhury and Kennedy, 1980). It is thought that beetles that are not yet responsive to attractive semiochemicals contain higher levels of lipids and that these individuals become responsive through a metabolic feedback process activated through flight exercise (Atkins, 1966, 1969). We hypothesize that a period of lipid metabolism through flight exercise may also be necessary before pheromone production can reach its maximum. In this way healthy, lipid-rich beetles could disperse farther from trees in which they developed before being able to produce large quantities of attractive pheromones. This longer dispersal would be of adaptive advantage, particularly under epidemic conditions, as these individuals would be more likely to encounter suitable hosts, thus assuring an adequate supply of high-quality phloem in which to feed and breed. A long dispersal flight would also enable individuals to interbreed with beetles from other populations. Older, wild beetles (reared as pupae and held 35–40 days posteclosion), which show much higher levels of pheromone production than wild emerged beetles (Figure 1), may be metabolically similar to emerged beetles that have completed a dispersal flight. They have probably lost lipid reserves through walking exercise in the Petri dishes and also may have faced a low-quality diet due to drying out of the phloem on which they were fed. Sanders (1983) showed that starvation or walking activity released host-positive responses in host-negative *Pityogenes chalcographus* (L.). Similarly, Gries (1984) showed that *I. typographus* that were denied a chance to fly became host-responsive only after incurring a mean weight loss equivalent to that lost during a 7-km flight. Therefore, we hypothesize that the older wild beetles shown in Figure 1 have foregone a dispersal flight due to decreasing lipid reserves and have become physiologically ready to attack new hosts, detoxify monoterpenes present in those hosts, and release aggregation pheromones at high levels.

When pupae were collected from infested bolts for use in the wild beetle maturation experiments, the beetles from two additional infested bolts from the same tree were allowed to emerge naturally. The average period of time between eclosion to adults in the Petri dishes and emergence of wild beetles from infested bolts was approximately 30 days. This is a very rough estimate of the time that adults wait before emerging in nature, since the infested bolts were held in cages

at approximately 27°C, and this elevated, constant temperature, in combination with the accelerated drying of the phloem, probably resulted in accelerated emergence. Nonetheless, this result supports the hypothesis that the 35-to-40-day posteclosion adults in Figure 1 were metabolically equivalent to emerged and dispersed adults, which would be expected to produce terpene alcohols at higher levels than emerged beetles.

An alternative explanation for 27-to-40-day posteclosion beetles producing higher levels of terpene alcohols than emerged beetles (Figure 1) is that the adults that were collected as pupae and allowed to mature on phloem strips may be microbe-deficient. Since these beetles did not feed *in situ* as callow adults, they may possess a microbial fauna that is substantially reduced or different from wild emerged individuals. Some of the microorganisms that would be present at reduced levels may utilize the terpene alcohols as substrates, metabolizing them into other products.

Although Figure 1 indicates that wild emerged beetles are apparently not capable of maximal terpene alcohol production, wild emerged beetles were used as a standard control for comparison in all subsequent experiments with axenically reared and streptomycin-fed beetles.

Effect of Maturation on Terpene Alcohol Production in Male I. paraconfusus. To test whether the physiological maturity of axenically reared *I. paraconfusus* also affects the beetle's ability to oxidize monoterpenes, males of varying posteclosion ages were exposed to α -pinene and myrcene vapors. Axenically reared individuals of five and 13 days posteclosion produced *trans*- and *cis*-verbenol and myrtenol at relatively low levels, while beetles of 18 and 26 days posteclosion produced these compounds at levels much higher than those in wild emerged beetles (Figure 2). Adults that were allowed to mature to 69 days posteclosion showed declining levels of terpene alcohol production. Wild beetles also matured in their ability to oxidize α -pinene (Figure 2), but, with the exception of myrtenol, did not exhibit the early peak in terpene alcohol production noted in young *D. ponderosae* adults (Figure 1). High levels of mortality prevented the testing of wild *I. paraconfusus* beyond 28 days posteclosion.

The peak levels of terpene alcohols produced by wild *I. paraconfusus* were only half of those produced by axenically reared beetles (Figure 2). In contrast to *D. ponderosae*, the peak levels of terpene alcohols produced by mature, wild *I. paraconfusus* did not exceed the production by wild emerged beetles (Figure 2). Oxidation products of myrcene, such as ipsdienol and ipsenol, were not generally detectable at quantifiable levels in either axenically reared beetles or wild controls exposed to myrcene vapors.

Axenically reared male *I. paraconfusus* of eight days posteclosion that were exposed to myrcene through feeding on bolts of *P. ponderosa* were unable to produce ipsenol and ipsdienol at measurable levels, while beetles of 24 and 32 days posteclosion produced these pheromones at levels similar to those in wild

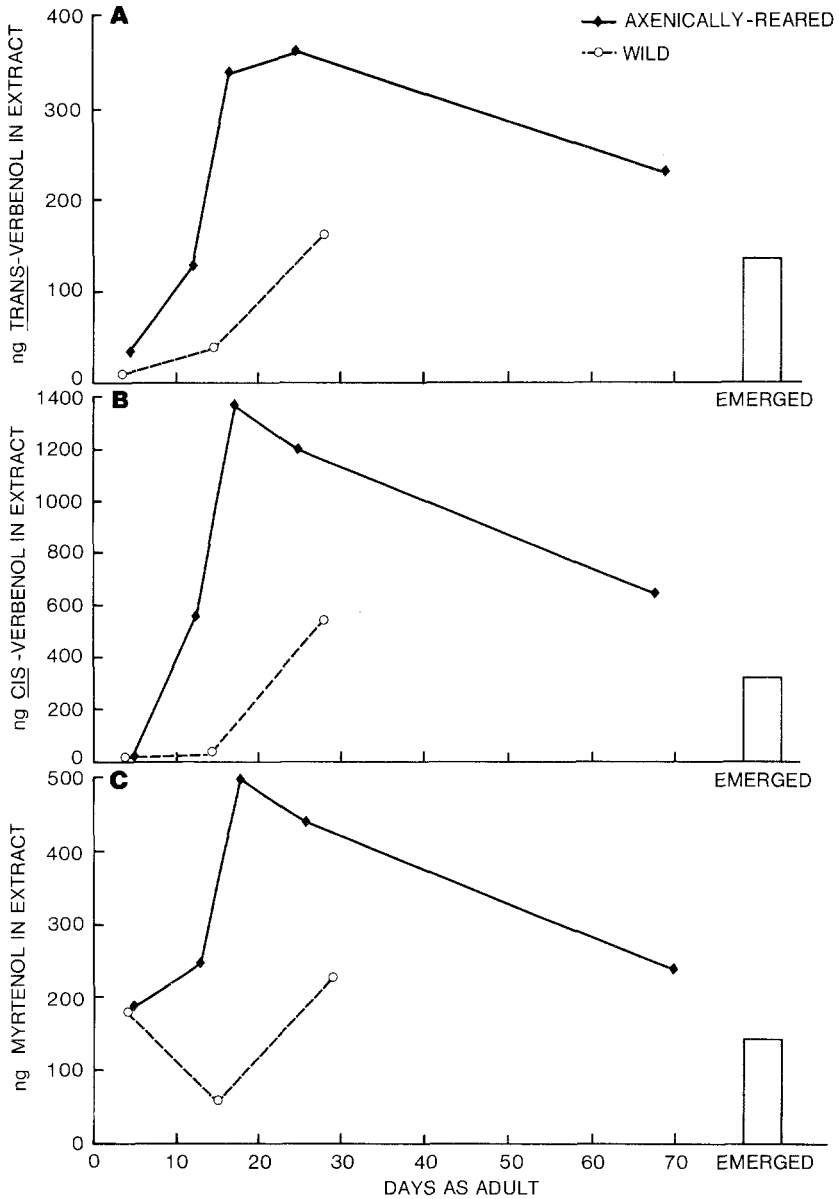


FIG. 2. Quantities (nanograms) of *trans*-verbenol (A), *cis*-verbenol (B), and myrtenol (C), produced in experiment XI by individual male *I. paraconfusus* of various posteclosion ages that were exposed to α -pinene vapors. Numbers of beetles used for each treatment were as follows. Axenically reared: 5 days, 10; 13 days, 11; 18 days, 12; 26 days, 11; 70 days, 10. Wild: 4 days, 7; 15 days, 5; 29 days, 5.

emerged beetles (Figure 3). When mature (24 days posteclosion), axenically reared individuals were fed on ponderosa pine bolts, they contained ipsenol and ipsdienol at levels that were not significantly different from those in mature wild beetles or wild emerged beetles (Table 3, experiment XIII).

As with *D. ponderosae*, Conn et al. (1984) did not report the posteclosion ages of *I. paraconfusus* used in their experiments. Although they did establish that axenically reared *I. paraconfusus* are capable of producing terpene alcohol pheromones, the generally low levels of production that they report probably

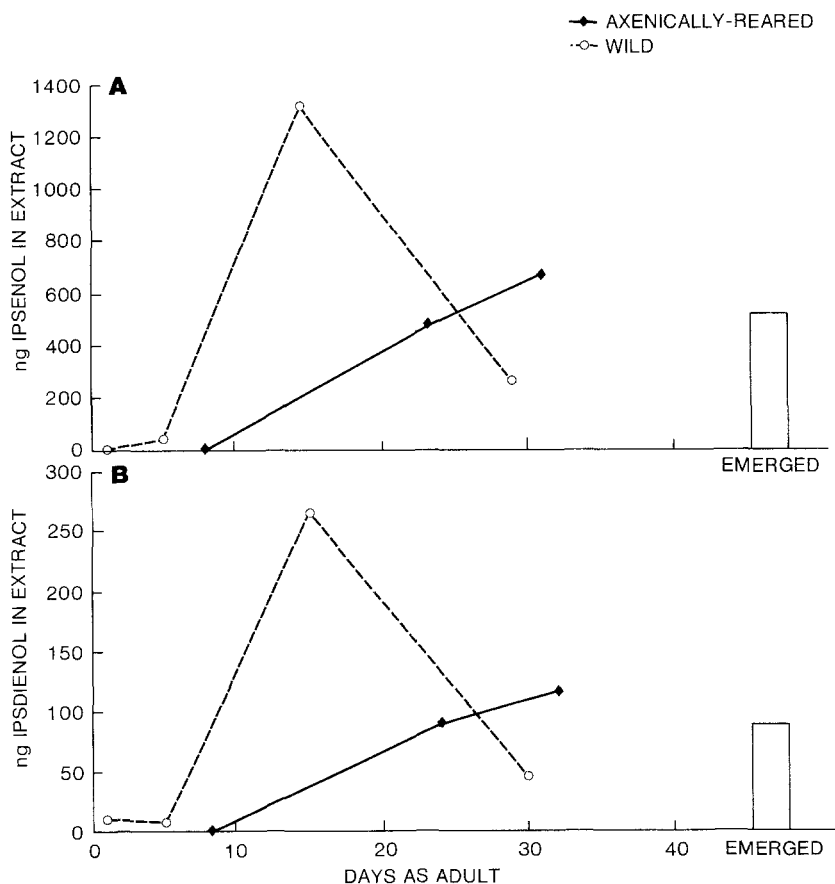


FIG. 3. Quantities (nanograms) of ipsenol (A), and ipsdienol (B), produced in experiment XII by individual male *I. paraconfusus* of various posteclosion ages that were fed on bolts of ponderosa pine. Numbers of beetles used for each treatment were as follows. Axenically reared: 8 days, 7; 24 days, 10; 32 days, 12. Wild: 1 day, 9; 5 days, 9; 15 days, 13; 30 days, 8.

reflect the use of axenically reared beetles that were not yet mature. The maturation period required before adult *I. paraconfusus* can convert myrcene into ipsenol and ipsdienol (Figure 3) and before adult *I. paraconfusus* and *D. ponderosae* can convert α -pinene into *cis*- and *trans*-verbenol and myrtenol (Figures 1 and 2) may explain why immature *I. paraconfusus* were unattractive to conspecifics when introduced into new hosts (Wood and Vité, 1961; Vité and Gara, 1962; Borden, 1967). It also may explain why immature female *D. frontalis* (Bridges, 1982) and *D. brevicomis* (Byers, 1983) were less capable of converting α -pinene to *trans*-verbenol than older females. Byers (1983) also found that immature male *I. paraconfusus* were not able to produce ipsenol and ipsdienol, although he reported that immature beetles were capable of producing normal amounts of *cis*- and *trans*-verbenol and myrtenol from α -pinene.

Microbial Involvement in α -Pinene Vapor Oxidation. Mature, axenically reared, female (Table 1, experiment IV) and male (Table 1, experiment IX) *D. ponderosae* produced more *trans*- and *cis*-verbenol and myrtenol upon exposure to α -pinene vapors than wild, emerged beetles. In addition, streptomycin-fed *D. ponderosae* females produced more of these terpene alcohols than wild, emerged beetles in one of two experiments (Table 1, experiments V and VI). Streptomycin-fed male *D. ponderosae* also produced more of these terpene alcohols than wild, emerged males (Table 1, experiment IX). Similarly, mature, axenically reared, male *I. paraconfusus* also produced more *trans*- and *cis*-verbenol and myrtenol upon exposure to α -pinene vapors than wild, emerged beetles (Table 3, experiment XIV). Mature, axenically reared, female *I. paraconfusus* (Table 3, experiment XXI), and streptomycin-fed male (Table 3, experiment XV) and female (Table 3, experiment XXI) *I. paraconfusus* also produced these terpene alcohols at levels not significantly different from wild, emerged beetles.

Evidently both male and female *D. ponderosae* and *I. paraconfusus* that have had their natural levels of symbiotic microorganisms reduced through axenic rearing or streptomycin feeding are capable of converting α -pinene vapors into the terpene alcohols *trans*- and *cis*-verbenol and myrtenol at levels equal to or significantly greater than those found in wild control beetles. These results confirm that readily culturable microorganisms are not solely responsible for the production of terpene alcohols in these beetles. The fact that terpene alcohol levels were significantly higher in microbe-reduced than in wild beetles in several experiments indicates that certain microorganisms may metabolize the available α -pinene precursor into other products, so that the α -pinene is unavailable to the beetles, or that they may utilize the terpene alcohols as substrates, metabolizing them into other products. In these ways the microorganisms present in wild bark beetles could regulate the levels of the aggregation pheromones, *trans*-verbenol in *D. ponderosae* and *cis*-verbenol in *I. paraconfusus*, preventing them from reaching excessive levels and disposing of them once aggregation

TABLE 3. OXYGENATED MONOTERPENE PRODUCTION IN AXENICALLY REARED, STREPTOMYCIN-FED, AND WILD *Ips paraconfusus* MALES (EXPERIMENTS XI–XVIII) AND FEMALES (EXPERIMENT XXI)

Exp. No.	Sex	Treatment	No. beetles	Mean amount of volatiles in abdominal extract (ng/beetle) ^a	
XIII	Males	Wild/emerged/air	9	ipsdienol <5 a	ipsenol <5 a
		Wild/emerged/ <i>P. ponderosa</i>	9	89 b	510 b
		Wild/mature/air	10	<5 a	<5 a
		Wild/mature/ <i>P. ponderosa</i>	13	264 b	1320 b
		Axenically reared/mature/air	10	<5 a	<5 a
XIV	Males	Axenically reared/mature/ <i>P. ponderosa</i>	10	92 b	478 b
		Wild/emerged/air	10	<i>trans</i> -verbenol <5 a	<i>cis</i> -verbenol <5 a
		Wild/emerged/ α -pinene and myrcene vapors	10	123 b	298 b
		Wild/mature/air	5	<5 a	<5 a
		Wild/mature/ α -pinene and myrcene vapors	5	159 bc	536 bc
XV	Males	Axenically reared/mature/air	11	<5 a	<5 a
		Axenically reared/mature/ α -pinene and myrcene vapors	12	336 c	1359 c
		Wild/air	10	<i>trans</i> -verbenol <5 a	<i>cis</i> -verbenol <5 a
		Wild/ α -pinene vapors	11	160 b	320 b
		Wild/streptomycin/air	9	<5 a	<5 a
XVI	Males	Wild/streptomycin/ α -pinene vapors	10	209 b	413 b
		Wild/air	12	<i>trans</i> -verbenol <5 a	<i>cis</i> -verbenol <5 a
		Wild/ <i>P. ponderosa</i>	6	8 a	12 a
		Wild/streptomycin/air	11	<5 a	<5 a
		Wild/streptomycin/ <i>P. ponderosa</i>	12	6 a	17 a

XVII	Males	Wild/ <i>P. ponderosa</i>	8	ipsdienol	ipsenol		
		Wild/new streptomycin/air	8	281 a	1529 a		
		Wild/new streptomycin/ <i>P. ponderosa</i>	8	<5 b	<5 b		
		Wild/old streptomycin/air	8	59 c	305 c		
		Wild/old streptomycin/ <i>P. ponderosa</i>	8	<5 b	<5 b		
				220 c			
XVIII	Males	Wild/air	10	<i>trans</i> -verbenol	<i>cis</i> -verbenol	myrtanol	ipsdienol
		Wild/ <i>P. ponderosa</i>	11	<5 a	<5 a	<5 a	<5 a
		Axenically reared/air	6	9 b	40 b	21 b	195 b
		Axenically reared/ <i>P. ponderosa</i>	11	<5 a	<5 a	<5 a	<5 a
				13 b	29 b	15 c	107 b
XIX	Males	Wild/air	10	<i>trans</i> -verbenol	<i>cis</i> -verbenol	myrtanol	ipsenol
		Wild/myrcene vapors	10	<5 a	<5 a	<5 a	<5 a
		Wild/ α -pinene and myrcene vapors	8	22 b	7 a	17 a	10 a
		Wild/ <i>P. ponderosa</i>	10	123 c	298 b	137 b	<5 a
				11 ab	9 a	19 a	54 b
XX	Males	Wild/myrcene vapors	10	ipsdienol	ipsenol		
		Wild/cellulose fed/myrcene vapors	11	<5 a	11 a	37 a	
XXI	Females	Wild/air	10	<i>trans</i> -verbenol	<i>cis</i> -verbenol	myrtanol	
		Wild/ α -pinene vapors	10	<5 a	<5 a	<5 a	
		Axenically reared/air	10	602 b	152 b	316 b	
		Axenically reared/ α -pinene vapors	11	<5 a	<5 a	<5 a	
		Wild/air/streptomycin	10	211 b	79 b	322 b	
				<5 a	<5 a		
		Wild/ α -pinene vapors/streptomycin	10	307 b	129 b	301 b	

^aData analyzed using the Kruskal-Wallis test followed by a nonparametric multiple comparisons test (Conover, 1980; p. 231), $P < 0.05$. Means within a column for each experiment followed by the same letter are not significantly different.

is complete. Alternatively, it is possible that reducing the levels of microorganisms in the beetles through axenic rearing or streptomycin feeding may reduce the levels of microorganisms that are mildly pathogenic to the beetles and that the resultant healthier beetles simply produce higher levels of terpene alcohols. In support of this hypothesis, Gueldner et al. (1977) found reduced levels of pheromone production in boll weevils, *Anthonomus grandis* Boheman that were artificially contaminated with *Streptococcus* sp., *Micrococcus varians* Migula, and *Enterobacter aerogenes* Hormaeche and Edwards, all isolated from apparently normal, insectary-reared weevils.

Two Sites and Two Methods of α -Pinene Oxidation. While axenically reared or streptomycin-fed *D. ponderosae* readily produced *trans*- and *cis*-verbenol and myrtenol from α -pinene vapors, axenically reared beetles were unable to produce *trans*-verbenol from ingested α -pinene (Table 1, experiment VII). This result suggests that there are two production systems for this pheromone, one by the beetle's own enzymes and one by symbiotic microorganisms. The low levels of *trans*-verbenol in extracts of wild beetles fed on lodgepole pine probably result from the conversion of α -pinene by microorganisms in the gut. The high levels of *trans*-verbenol, *cis*-verbenol, and myrtenol in extracts of axenically reared and streptomycin-fed *D. ponderosae* exposed to α -pinene vapors would then be due to oxidation of inhaled vapors by the beetle's own enzymes.

Since axenically reared *D. ponderosae* produce *trans*-verbenol from inhaled, rather than ingested α -pinene (Table 1, experiments IV and VII), it is likely that the conversion does not occur in the gut, as suggested for other scolytids (Pitman et al., 1965; Zethner-Møller and Rudinsky, 1967). *trans*-Verbenol is probably produced in the hemolymph, transported through the Malpighian tubules into the hindgut, and released through the anus. This explanation is consistent with Hughes' (1973) finding of *trans*-verbenol and other monoterpene oxidation products in the hemolymph of *D. ponderosae* and his hypothesis that terpene metabolism occurs outside the gut.

Wild female *D. ponderosae* that had fed in lodgepole pine bolts for 24 hr contained significant amounts of verbenone, while axenically reared individuals contained only trace quantities (Table 1, experiment VII). However, after exposure to α -pinene vapors, neither wild nor axenically reared female *D. ponderosae* contained quantifiable levels of verbenone (Table 1, experiment IV), even when the extracts were concentrated to approximately 5 μ l over a stream of nitrogen before gas chromatographic analysis. Similarly, exposure to α -pinene vapors does not result in verbenone production by *D. frontalis* (Renwick et al., 1973), or *D. brevicomis* (Byers, 1983). Therefore, the production of verbenone by female *D. ponderosae* is probably due solely to the conversion of beetle-ingested precursor by gut microorganisms. This suggests that *D. ponderosae* is dependent on its symbiotic microorganisms for the production of verbenone, its

principal antiaggregation pheromone (Ryker and Yandell, 1983; Libbey et al., 1985; Borden et al., 1987).

Microbial Involvement in Myrcene Oxidation. Both axenic rearing and streptomycin feeding reduced the levels of (*E*)-1-myrcenol (1-myrcenol = 2-methyl-6-methylene-octa-2,7-dien-1-ol) and an unknown product in male *D. ponderosae* exposed to myrcene vapors (Table 1, experiment X). The levels of (*Z*)-1-myrcenol were reduced by streptomycin feeding, while the reduction caused by axenic rearing was not significant. This is further evidence that microorganisms are partially responsible for the production of certain conversion products of monoterpenes in this species. However, streptomycin-fed and wild control females exposed to myrcene vapors for 24 hr produced (*Z*) and (*E*)-1-myrcenol and an unknown product at similar levels (Table 1, experiment VIII).

Axenically reared or streptomycin-fed male *D. ponderosae* that were subsequently exposed to myrcene vapors contained ipsdienol at levels that were not significantly different from those in wild, emerged beetles (Table 1, experiment X). None of the females tested produced detectable levels of ipsdienol (Table 1, experiment VIII).

Two Pheromone Production Systems. The hypothesis that both the beetle and its symbiotic microorganisms can produce *cis*- and *trans*-verbenol, as well as other terpene alcohols, is consistent with the report by Chararas et al. (1980) that the frass of *I. sexdentatus*, *I. typographus*, or *I. acuminatus* that had been fed a wide-spectrum antibiotic showed reduced attractiveness to conspecifics. Chararas et al. (1980) also reported that the frass of antibiotic-fed beetles had more α -pinene, β -pinene, and Δ_3 -carene and less of their conversion products than normal beetles. They concluded that microbial conversion was responsible for a portion of total pheromone production, but provided minimal methodological details and presented no data to support this conclusion.

The existence of two pheromone production systems, beetle and microbial, is also consistent with what is known about polysubstrate monooxygenases (PSMOs). PSMOs are enzymes that catalyze numerous oxidation reactions that render lipophilic compounds, such as monoterpenes, hydrophilic, so that they can be more easily excreted. They are likely the most important enzymes involved in the metabolism of foreign substances, such as monoterpenes (Sturgeon and Robertson, 1985), and compounds that inhibit PSMO activity have been shown to inhibit partially terpene alcohol pheromone production in *D. ponderosae* (Hunt and Smirle, 1988). Since PSMOs are believed to be present in all aerobic organisms (Brattsten, 1979), and since bark beetles and their microorganisms are frequently exposed to monoterpenes that are toxic to both the insects (Smith, 1965; Reid and Gates, 1970; Coyne and Lott, 1976; Raffa and Berryman, 1983) and their symbiotic microorganisms (Cobb et al., 1968;

Shrimpton and Whitney, 1968; DeGroot, 1972; Raffa et al., 1985), it is logical that both would use PSMOs to oxidize monoterpenes.

Effect of Streptomycin on I. paraconfusus. In experiment XVI (Table 3) streptomycin feeding resulted in virtual elimination of the production of ipsdienol and ipsenol, a result in agreement with those of Byers and Wood (1981). However, in experiment XVII (Table 3), streptomycin-feeding caused a significant, but not total, reduction in ipsdienol and ipsenol production. Although the streptomycin used in these two experiments was from the same batch, there were several months between the experiments. Therefore, newly purchased streptomycin was used to determine whether the streptomycin in experiment XVII had lost some of its activity. However, the levels of ipsenol and ipsdienol in beetles fed the old or the new streptomycin were not significantly different (Table 3, experiment XVII).

Our *I. paraconfusus* colony was initiated with beetles from the Sierra Nevada, and should have been very similar to those used by Byers and Wood (1981). We had reared these beetles through several generations by the time experiment XVII (Table 3) was done, and it appears that this resulted in a shift of the population of insects or their symbiotic microorganisms toward some characteristic that promoted resistance to the effects of streptomycin.

Streptomycin Feeding vs. Axenic Rearing. There was only one discrepancy between the results obtained using axenically reared or streptomycin-fed beetles. The levels of ipsenol and ipsdienol in axenically reared and wild male *I. paraconfusus* were not significantly different (Table 3, experiments XIII and XVIII). However, streptomycin-feeding of male *I. paraconfusus* significantly reduced the production of these terpene alcohols (Table 3, experiments XVI and XVII), as previously shown by Byers and Wood (1981). This anomaly could indicate that the reduction in ipsenol and ipsdienol production due to streptomycin feeding may be caused by toxic effects on insect cells rather than the elimination of certain microorganisms. This hypothesis appears to be somewhat unlikely since the production of *cis*- and *trans*-verbenol and myrtenol is not affected by streptomycin-feeding. Alternatively, the conversion of myrcene to ipsenol and ipsdienol could be performed by symbiotic microorganisms that are eliminated by streptomycin feeding but not by axenic rearing. These microorganisms would have to be transovarially transmitted, obligate symbionts to avoid elimination through axenic rearing and detection during the culturing of axenically reared beetles. Some evidence for transovarial transmission of bacteria in bark beetles has previously been reported (Buchner, 1965).

Vapors vs. Feeding—"Contact" and "Frass" Pheromones. In most bark beetles, myrcene is converted to ipsdienol and ipsenol more efficiently if the exposure is through feeding, while α -pinene is converted to *cis*- and *trans*-verbenol and myrtenol more efficiently following exposure to vapors (Vité et al., 1972). Experiment XIX (Table 3) confirms that this trend is also true for *I.*

paraconfusus. When males were exposed to α -pinene and myrcene vapors simultaneously, conversion of α -pinene to *trans*- and *cis*-verbenol and myrtenol was much more efficient than conversion of myrcene to ipsenol and ipsdienol. When beetles were exposed only to myrcene vapors, slightly more ipsenol and ipsdienol were produced, but the effect was not significant. However, when beetles were exposed by feeding to the myrcene and α -pinene in ponderosa pine bolts, the conversion of myrcene to ipsenol and ipsdienol was much more efficient than with vapor exposure, while the conversion of α -pinene was significantly less efficient (Table 3, experiment XIX). As a result, experiments with *I. paraconfusus* generally used vapor exposure to study the conversion of α -pinene, and feeding to study the conversion of myrcene.

Hughes and Renwick (1977) proposed that the production of ipsenol and ipsdienol by newly emerged *I. paraconfusus* is prevented by neural inhibition that is removed by distension of the gut during natural feeding. Hughes and Renwick (1977), working with *I. paraconfusus*, and Harring (1978), working with *Pityokteines curvidens* Germ. and *Pityokteines spinidens* Reit., were able to induce the production of ipsenol and ipsdienol by distending the guts of insects with an injection of air before exposing them to myrcene vapors. This approach was criticized by Byers (1981), who suggested that the distension of the gut with air would enhance the diffusion of myrcene vapors in the gut, thus promoting pheromone synthesis artificially by increasing precursor diffusion to the site of synthesis. Our approach to this problem was to distend the guts of *I. paraconfusus* by feeding them with powdered cellulose and then expose the beetles to myrcene vapors. This treatment resulted in a 3.5-fold increase in ipsenol content and a slight increase in ipsdienol content compared to controls (Table 3, experiment XX). However, these differences were not significant due to the large numbers of zeros in the data.

D. ponderosae also convert α -pinene more efficiently through vapor exposure than through feeding (Table 1). Female beetles fed on lodgepole pine phloem produced very little *cis*- and *trans*-verbenol and myrtenol (Table 1, experiment VI), while beetles exposed to α -pinene vapors produced much larger quantities of these terpene alcohols (Table 1, experiment VI). In contrast to *I. paraconfusus*, male *D. ponderosae* exposed to myrcene vapors produced large quantities of ipsdienol, as well as (*Z*)- and (*E*)-1-myrcenol (Table 1, experiment X), while males fed on bolts of lodgepole pine did not produce these products in quantifiable amounts.

Since myrcene is generally converted more efficiently through feeding and α -pinene is converted more efficiently through vapor exposure, ipsdienol has been termed a "frass" pheromone, while *cis*- and *trans*-verbenol are termed "contact" pheromones (Vité et al., 1972). Our data, as well as those of Byers (1982) and Hughes (1974), indicate that in *Dendroctonus* species myrcene is actually oxidized much more efficiently with vapor exposure than with feeding.

This conclusion is in agreement with the hypothesis presented by Vité et al. (1972), suggesting that aggressive bark beetles, such as *D. ponderosae*, begin oxidizing monoterpenes upon initial contact with a new host, while less aggressive species, such as many *Ips* species, depend on feeding for the conversion of monoterpenes. Aggressive bark beetle species, which attack healthy, resinous trees, must use rapid aggregation in mass-attacking their hosts to overcome host tree resistance. It is therefore logical that they would depend on rapidly produced contact pheromones. Less aggressive species, which generally attack trees with weakened or nonexistent defenses, can postpone mass attack until pioneer individuals have established, through feeding and defecation, that the host phloem is of suitable quality.

CONCLUSIONS

We have found that physiological maturation is required before axenically reared bark beetles can produce terpene alcohol pheromones maximally from monoterpenes. Our data provide a rationale for the extended maturation feeding by bark beetles prior to emergence from their brood host. Premature emergence would result in an unfit beetle incapable of producing optimal amounts of pheromone.

Since the reduction in levels of microorganisms in *I. paraconfusus* through antibiotic feeding reduces the production of ipsenol and ipsdienol, while axenic rearing or antibiotic feeding of *D. ponderosae* does not reduce the production of terpene alcohols, it appears that *I. paraconfusus* is more dependent on its symbiotic microorganisms than is *D. ponderosae*. Because it is a very aggressive bark beetle, *D. ponderosae* requires a very active enzyme system to rapidly detoxify the monoterpenes encountered in the hosts, as well as to produce large quantities of aggregation pheromones rapidly in order to attract enough individuals to overcome host resistance. However, *I. paraconfusus* generally breeds in fallen or cut trees (S.L. Wood, 1982), which have reduced levels of monoterpene-rich resin. These beetles do not require as efficient an enzyme system because the host environment is less toxic than that for *D. ponderosae* and also because a less rapid and less efficient production of aggregation pheromones is adequate in overcoming host resistance. Thus *I. paraconfusus* can rely partially on microorganisms for the production of terpene alcohols, while *D. ponderosae* is apparently capable of performing this conversion without microorganisms.

Although the production of normal or elevated levels of pheromones and other terpene alcohols by axenically reared *D. ponderosae* and *I. paraconfusus* does not rule out the possibility that symbiotic microorganisms may be involved in the production of these compounds, it does indicate that readily culturable microorganisms are not required for this process. Since the reports in the lit-

erature that describe the production of coleopteran pheromones in vitro by their symbionts (Hoyt et al., 1971; Brand et al., 1975, 1976; Chararas et al., 1980) involve readily culturable microorganisms, our conclusion is that there is no direct evidence that symbiotic microorganisms are required for pheromone production in the Coleoptera, with the exception of the antibiotic feeding studies conducted here and by Byers and Wood (1981). Our data do add support to the assertion that certain microorganisms are capable of oxidizing monoterpenes, but establish that *D. ponderosae* and, to a certain extent, *I. paraconfusus* are capable of producing large quantities of terpene alcohol pheromones in the absence of readily culturable microorganisms.

In contrast to the aggregation pheromones and other terpene alcohols examined during this study, there is considerable evidence for significant microbial involvement in the production of the antiaggregation pheromone verbenone by *D. ponderosae*. This ketone was not produced in quantifiable levels by axenically reared or streptomycin fed beetles that were exposed to α -pinene as vapors or through feeding. However, significant quantities of verbenone were found in wild *D. ponderosae* that were exposed to α -pinene through feeding on bolts of lodgepole pine. This result suggests that verbenone is not formed by the beetle, but is produced from *trans*-verbenol by microorganisms in its gut. This hypothesis is supported by reports that yeasts capable of converting verbenols to verbenone have been isolated from *D. ponderosae* (Hunt and Borden, 1989) and *I. typographus* (Leufvén et al., 1984). We hypothesize that similar oxidative metabolism of terpene alcohols by microorganisms may be responsible for the significantly lower levels of these compounds in wild beetles than in microbe-reduced beetles. In this way microorganisms would regulate pheromone levels in the host insects.

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STRAWBERRY RESISTANCE TO *Tetranychus urticae*
KOCH: EFFECTS OF FLOWER, FRUIT, AND
FOLIAGE REMOVAL—COMPARISONS OF
AIR- VS. NITROGEN-ENTRAINED
VOLATILE COMPOUNDS

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Abstract—An increase in resistance to the two-spotted spider mite (TSSM), *Tetranychus urticae* Koch, is observed in field-grown strawberry plants during the period from flowering to postharvest. This seasonal phenomenon was investigated to determine the influence of the metabolic sink, that is, fruiting in the plant. Removal of flowers and fruit and partial removal of foliage did not alter the pattern of resistance of the strawberry plant to TSSM. Bioassays were conducted in concert with chemical analyses. Headspace chemicals emitted from foliage samples were entrained in air and trapped on Tenax, identified, and compared with those entrained in nitrogen and trapped. Terpenes were among the major compounds entrained in air, whereas alcohols were obtained with nitrogen. The air-entrained headspace compounds did not appear to correlate quantitatively with the development of mite resistance in the control plants or those subjected to metabolic sink (flower and fruit) removal. Evidence was obtained for the presence of heretofore unreported strawberry foliage headspace components, namely, (Z)-3-hexenyl 2-methylbutyrate, (Z)-3-hexenyl tiglate, (E)- β -ocimene, (Z)- β -ocimene, α -farnesene, and germacrene D.

Key Words—*Tetranychus urticae*, two-spotted spider mite, Acari, Tetranychidae, strawberry resistance, metabolic sink removal, volatile foliage compounds, headspace entrainment.

INTRODUCTION

Strawberry plants develop seasonally correlated resistance to the two-spotted spider mite (TSSM) *Tetranychus urticae* Koch (Shanks and Barritt, 1975; Rodriguez and Rodriguez, 1987). Plants are susceptible to mite attack in early spring, during prebloom and flowering, but become resistant after fruit harvest in June. Based on the observation that resistance to TSSM increases after fruit development and harvest, the question arose as to the possible relationship of fruit development and maturation to the phenomenon of resistance. It was postulated that the development of flowers and fruit acts as a metabolic sink that may change metabolic patterns in the leaves and thereby alter resistance of the plant to mites (Hamilton-Kemp et al., 1988). Accordingly, we decided to remove flowers, fruit, and foliage from strawberry plants to determine whether the development of resistance in plants would be affected. Previous studies were made to determine the profile of volatile compounds emitted by the foliage (during resistance development) using nitrogen as the entrainment gas (to prevent oxidation). Recently, Buttery et al. (1987) demonstrated that aerobic conditions (air entrainment) could be used to collect volatiles with good recovery of compounds representing different chemical classes. In the present studies, air-entrained volatiles were compared with those compounds entrained with nitrogen and the effect of metabolic sink removal on volatiles was examined.

METHODS AND MATERIALS

Plant Material. Redchief strawberry plants (*Fragaria ananassa* Duch.) were grown in field plots at the University of Kentucky farm in Lexington. Standard cultural practices were followed, but pesticides were not applied to the plants. Treatments were in a randomized complete block with four replicates. Plants were treated in the following ways: (A) control, normal flowering and fruiting were allowed to proceed; (B) inflorescence was removed at first bloom; (C) all fruit was removed two weeks after first bloom; (D) two thirds of the foliage (two lateral trifoliate leaflets) was removed from plants two weeks after first bloom and fruiting was permitted; (E) two thirds of the foliage was removed two weeks after first bloom and fruiting was not permitted. Fruit was harvested when ripe in treatments A and D.

Bioassay. The detached leaf disk bioassay was carried out as described previously (Hamilton-Kemp et al., 1988). Leaves were collected for bioassay on five different sampling dates in 1987: April 28, May 12, May 19, June 3, and June 17, which corresponded to flowering, fruit initiation, start of fruit harvest, completion of fruit harvest, and two weeks postharvest for control plants, respectively. Five leaflets from each of four field replicates were collected for bioassay, making a total of 20 leaflets per sampling date for each treatment. Analysis of variance and Duncan's multiple-range test were performed on the data.

Headspace Analyses. Headspace analyses were carried out using Tenax traps (Hamilton-Kemp, et al., 1988) except that harvested leaves were analyzed intact rather than being cut into segments. Air and nitrogen (both high purity, compressed) were used as entrainment gases. For comparison of volatiles entrained by air and those entrained by nitrogen, leaflets were harvested in mid-June and samples were divided into two 100-g portions and analyzed simultaneously. Comparisons of air-entrained volatiles were made at different times during the season. Plants studied included those that were allowed to develop fruit normally and those that were not allowed to fruit. Harvesting was done in late April, mid-May, and mid-June, which corresponded to the following stages of development on control plants: flowering, fruiting, and two weeks after fruit harvest. All headspace sampling experiments described above were carried out three times, except those at flowering which were done twice.

Compound Identification and Quantitation. Compounds were analyzed by GC using a 60-m \times 0.32-mm ID Carbowax 20 M column and GC-MS as described previously (Hamilton-Kemp et al., 1988). Spectra and GC retention data were compared with those of compounds obtained from commercial suppliers or from Tenax-trapped headspace samples that we prepared from previously established plant sources. (*E*)- and (*Z*)- β -Ocimene were obtained from clover foliage (Buttery et al., 1984); α -farnesene and germacrene D were obtained from ylang ylang oil (Buttery et al., 1986). (*Z*)-3-Hexenyl 2-methylbutyrate and (*Z*)-3-hexenyl tiglate were obtained from Bedoukian Inc., Danbury, Connecticut. Kovats' indices for headspace compounds were determined following the method of Perry (1981).

Following adsorption of volatiles on a Tenax trap, compounds were removed for quantitation by extraction with three 10-ml portions of hexane. The hexane extracts were combined and concentrated to approximately 1 ml in a microstill equipped with a Vigreux column. To the concentrated solution, 1.67 μ g of cumene was added as an internal standard. An aliquot of the concentrated solution was analyzed by GC, and peak areas of leaf volatiles relative to that of the internal standard were determined. Data were analyzed by analysis of variance and Duncan's multiple-range test.

RESULTS AND DISCUSSION

Metabolic Sink Removal and TSSM Resistance. Results of the bioassays carried out to determine the effect of sink removal and stress induced by defoliation on TSSM resistance of plants are presented in Table 1. Plants with flowers and fruits removed showed increased vegetative growth, which was manifested in increased size of leaflets. A steady increase in mite resistance developed in all plants and treatments until the final sampling period in mid-June, which was about two weeks after fruit harvest in control plants. The resistance was observed for both the immature forms and the adult forms of TSSM (Table 1). This pattern of resistance development is well documented (see review paper Rodriguez and Rodriguez, 1987). The present study also demonstrated that removal of metabolic sinks (flowers and fruits) did not generally alter the expression of high seasonal resistance of strawberry plants to mites.

In the studies of the effects of defoliation stress, it was expected that partial defoliation of the plants would result in a reduction of plant vigor and perhaps alter the ability of the remaining foliage to resist mite infestation. However, the

TABLE 1. NUMBER OF TSSM SEVEN DAYS AFTER PLACING FIVE TENERAL FEMALES ON LEAFLETS FROM DEFLOWERED, DEFRUITED, AND PARTIALLY DEFOLIATED STRAWBERRY PLANTS

Treatment ^a	Date of sampling				
	April 28	May 12	May 19	June 3	June 17
Eggs and immatures ^b					
A	201a	98.2a	49.3a	0a	0a
B	147a	63.3ab	12.4b	3.0a	0a
C	169a	58.7b	42.3a	0.3a	0a
D		65.2ab	55.4a	17.8a	0.05a
E		90.0ab	32.2ab	12.8a	0a
Adults ^b					
A	3.65a	3.25a	1.55abc	0c	0a
B	3.05a	2.05b	0.75c	0.60bc	0a
C	3.20a	1.85b	1.60ab	0.20c	0a
D		2.85a	2.05a	1.45a	0.05a
E		3.00a	1.60a	1.05ab	0a

^aTreatments: A—control with normal fruiting; B—flowers removed at first bloom; C—fruit removed two weeks after B; D—defoliated $\frac{2}{3}$ at final bloom, normal fruiting; E—defoliated $\frac{2}{3}$ at final bloom, fruit removed.

^bMeans in each column followed by the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple-range test.

bioassays showed that the remaining foliage on both treatments developed resistance to mites (Table 1).

Hence, the treatments selected to remove the metabolic sink and prevent normal ontogeny of fruit and to induce physiological stress through defoliation of strawberry plants did not markedly change the general seasonal resistance trends observed in previous studies. The bioassay results show that all plants tested became resistant to TSSM over the period from flowering in early spring to after fruit harvest in mid-June.

Air vs. Nitrogen Headspace Entrainment and New Compounds. Air was used as entrainment gas in the present experiments and compared to nitrogen entrainment of strawberry volatiles used previously (Hamilton-Kemp et al., 1988). Qualitative and quantitative analytical differences were obtained with the two gases (Table 2). Notably, there were more types, and larger quantities,

TABLE 2. COMPARISON OF STRAWBERRY FOLIAGE VOLATILES ENTRAINMENT BY AIR AND NITROGEN AND TRAPPED ON TENAX

Compound ^a	Amount (ppb \pm SE) ^b	
	Air	Nitrogen
(Z)- β -Ocimene	15 \pm 6a	4 \pm 6a
Hexyl acetate	nd	8 \pm 1
(E)- β -Ocimene	376 \pm 65a	42 \pm 65b
(Z)-3-Hexenyl acetate	67 \pm 32a	318 \pm 32b
1-Hexanol	nd	124 \pm 10
(Z)-3-Hexen-1-ol	24 \pm 500a	2608 \pm 500b
1-Octen-3-ol	nd	103 \pm 1
6-Methyl-5-hepten-1-ol	nd	43 \pm 1
(Z)-3-Hexenyl 2-methylbutyrate	tr a	38 \pm 1b
Linalool	nd	131 \pm 5
(Z)-3-Hexenyl tiglate	nd	34 \pm 2
α -Terpineol	nd	8 \pm 0
Germacrene D	254 \pm 51	nd
Sesquiterpene	23 \pm 5a	3 \pm 5a
α -Farnesene	157 \pm 31	nd
Methyl salicylate	nd	337 \pm 35
Ethyl salicylate	nd	40 \pm 0
Benzyl alcohol	2 \pm 7a	304 \pm 7b
2-Phenethyl alcohol	nd	53 \pm 13

^a Order of increasing retention on Supelcowax 10. (E)-2-Hexenal was detected but not quantitated due to an interfering peak.

^b Means followed by the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple-range test. nd = not detected; electronic integrator did not list area value for compound. tr = trace, integrated less than 1 ppb.

of alcohols and their esters obtained in a nitrogen than in an air atmosphere. The formation of volatile alcohols may have been due to higher activities of alcohol dehydrogenase enzymes, which have been found under anaerobic conditions in various plant species (Davies, 1980). These enzymes may effectively reduce aldehydes to their corresponding alcohols. Phenolic salicylates also were found in nitrogen-entrained isolates; they increased after fruit harvest (Hamilton-Kemp et al., 1988), which may indicate a generally increased potential for formation of phenolic compounds in late-season plants. Nonvolatile phenolic compounds have been associated with plant resistance to certain insects, as discussed elsewhere (Feeny, 1976; Stipanovic, 1983).

During air entrainment, terpene hydrocarbons were emitted, and these comprised a major part of the volatiles found. Mass spectral and chromatographic evidence was obtained for *E* and *Z* isomers of β -ocimene and the sesquiterpene hydrocarbons α -farnesene and germacrene D (Tables 2 and 3). Another major sesquiterpene was isolated that could not be identified from available spectra. The fact that these compounds were found in higher levels in air may indicate that they turn over rapidly and/or are not generated effectively in nitrogen.

In addition to the terpene hydrocarbons, the results from interpretation of mass spectra and subsequent comparison with authentic compounds provided evidence for infrequently reported esters of leaf alcohol [(*Z*)-3-hexen-1-ol], namely (*Z*)-3-hexenyl 2-methylbutyrate and (*Z*)-3-hexenyl tiglate. These compounds probably form from the high available levels of leaf alcohol that can be esterified with an assortment of acids that occur free in the strawberry plant.

Air-Entrained Volatiles and Metabolic Sink Removal. Greater quantities of compounds were emitted from young actively growing plants in late April dur-

TABLE 3. KOVATS' AND MASS SPECTRAL EVIDENCE FOR NEWLY IDENTIFIED COMPOUNDS FOUND IN HEADSPACE FROM STRAWBERRY FOLIAGE

Compound	Kovats' index ^a	Mass spectral peaks ^b	Molecular weight
(<i>Z</i>)- β -Ocimene	1248	93, 92, 91, 79, 41, 77, 53	136
(<i>E</i>)- β -Ocimene	1266	93, 91, 79, 80, 77, 41, 92	136
(<i>Z</i>)-3-Hexenyl 2-methylbutyrate	1491	67, 82, 57, 85, 41, 55, 83	184
(<i>Z</i>)-3-Hexenyl tiglate	1688	67, 82, 83, 55, 41, 53, 54	182
Germacrene D	1735	161, 105, 91, 41, 79, 81, 77	204
α -Farnesene	1763	41, 93, 69, 55, 79, 91, 107	204
Sesquiterpene	1746	93, 41, 119, 69, 91, 55, 79	204

^aDetermined for Supelcowax 10 column.

^bIons above *m/z* 40 in decreasing order of intensity.

ing flowering than from plants analyzed at other times (Table 4). Furthermore, several compounds present during this period were not detected or were present only in trace amounts at later sampling times. There were numerous, primarily small, peaks in the samples, which were not identified, even though the major components entrained by air were identified.

Generally, there were no statistically significant differences between the volatiles in control and deflowered plants. Thus, the removal of the metabolic sink did not markedly affect emissions of foliage headspace compounds. There did not appear to be consistent changes in air-entrained volatiles from control or deflowered plants, which corresponded with the steady increase in plant resistance to mites that was observed.

Clearly, there was considerable variation in the quantitative data obtained and used in comparisons. This may have been partially due to the small quantities of compounds emitted by the plant samples. Other investigators also have noted considerable variation in quantities of volatiles obtained from one sampling to another (Buttery et al., 1987). We observed in one case that there was a decrease in overall yield of volatiles obtained from strawberry foliage within 12 hr following heavy rainfall on field-grown plants. The precipitation may

TABLE 4. STRAWBERRY FOLIAGE VOLATILES ENTRAINED BY AIR AND TRAPPED ON TENAX AT PERIODS OF TSSM RESISTANCE DEVELOPMENT IN CONTROL (FRUITING) AND DEFLOWERED (NONFRUITING) PLANTS

Compound	Amount (ppb \pm SE) ^a				
	Late April flowering	Mid-May control	Mid-May deflowered	Mid-June control	Mid-June deflowered
(Z)- β -Ocimene	15 \pm 3a	tr	7 \pm 2b	7 \pm 2b	2 \pm 2b
(E)- β -Ocimene	68 \pm 39a	7 \pm 31b	44 \pm 31ab	120 \pm 31a	75 \pm 31a
Hexyl acetate	21 \pm 1a	1 \pm 1b	3 \pm 1b	tr	tr
(Z)-3-Hexenyl acetate	2475 \pm 122a	465 \pm 100bc	624 \pm 100b	164 \pm 100c	224 \pm 100c
1-Hexanol	5 \pm 1a	tr	2 \pm 1b	tr	tr
(E)-2-Hexenyl acetate	19 \pm 1a	nd	1 \pm 1b	tr	nd
(Z)-3-Hexen-1-ol	106 \pm 2a	35 \pm 17b	56 \pm 17ab	40 \pm 17b	59 \pm 17ab
(Z)-3-Hexenyl 2-methylbutyrate	9 \pm 0	nd	nd	nd	nd
(Z)-3-Hexenyl tiglate	54 \pm 2a	nd	2 \pm 2b	nd	nd
Germacrene D	35 \pm 17ab	1 \pm 14b	17 \pm 14ab	62 \pm 14a	21 \pm 14ab
Sesquiterpene	22 \pm 5a	3 \pm 4b	12 \pm 4ab	11 \pm 4ab	2 \pm 4b
α -Farnesene	191 \pm 34a	5 \pm 28b	74 \pm 28b	95 \pm 28b	40 \pm 28b

^aMeans in each row followed by the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple-range test. Flowering plants (first column) were sampled twice; the remaining plants (last four columns) were analyzed three times each. tr = trace, less than 1 ppb. nd = not detected, electronic integrator did not list area value for compound in sample.

have washed volatiles from the foliage or prevented movement of volatiles from the sources of emission on the tissue.

From the present research using air entrainment of volatiles, there did not appear to be direct correlations with the strawberry volatile composition and development of resistance to TSSM. The appearance and increase in salicylates during the growing season seen in the earlier studies (Hamilton-Kemp et al., 1988) using nitrogen may have been an effect caused by the anerobic nitrogen atmosphere. However, the increase in salicylates over the growing season may indicate an increased potential for phenolic compound synthesis as the plants aged during the season (as discussed earlier).

In summary, the experimental results showed that foliage resistance to immature and adult forms of TSSM continued to develop even though the metabolic sinks, namely flowers and fruits, were removed and plants were subjected to partial defoliation. It was evident that the factors that caused the expression of resistance were not tied to the normal ontogeny of fruiting and were not modified by physiological stresses associated with defoliation. One environmental factor that may be pertinent for future study is day length, as photoperiod occurring in the field over the fruiting season could initiate the development of plant resistance to TSSM. Past preliminary experiments indicated that the development of resistance in greenhouse-grown plants (in contrast to field-grown plants) did not occur in a predictable pattern, and this observation may be consistent with lack of control of day length. It also was clear from strawberry foliage analyses that the patterns of volatiles obtained in air were different from those obtained in nitrogen. Metabolic sink removal did not significantly change volatile emissions from the foliage. Although these compound types have been postulated to be factors in the interaction of plants with certain insects (cf. Siegler, 1983; Finch, 1986), there does not seem to be a correlation between volatiles entrained by air and the development of strawberry resistance to mites. Foliage volatiles may play a role in strawberry interactions with other insects and pathogens as has been observed in other plant species.

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BIOLOGICAL ACTIVITY OF SYNTHETIC
HYDROCARBON MIXTURES OF
CUTICULAR COMPONENTS OF
THE FEMALE HOUSEFLY
(*Musca domestica* L.)

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Abstract—Mixtures of (*Z*)-9-alkenes (C₂₁–C₃₃), *n*-alkanes (C₂₁–C₃₃), and mono- and dimethylalkanes (C₂₇, C₂₉), as components of the cuticular lipids washed from the female housefly (*Musca domestica* L.), were synthesized and assayed for their biological activity on male houseflies. The (*Z*)-9-alkenes and their hydrogenated *n*-alkanes were synthesized from jojoba wax components by the appropriate chain elongation. The methylalkanes were prepared by Grignard coupling reaction of the appropriate alkyl halides, catalyzed by Li₂CuCl₄. Six- to 7-day-old virgin male houseflies exhibited the highest mating strike activity toward 6- to 7-day-old virgin females. The mating strike activity of the synthetic hydrocarbons was studied by exposing 6- to 7-day old virgin males to petrol-ether-rinsed 1 to 2-day-old dead females treated with these chemicals. (*Z*)-9-Tricosene was the most active hydrocarbon tested when it was applied in amounts of 10 μg to a washed dead female. Amounts of 5 μg of (*Z*)-9-tricosene did not cause sexual activity in the males. Mixture of (*Z*)-9-alkenes showed low activity when applied at 10-μg amounts. However, when mixtures were made of 5 μg of (*Z*)-9-tricosene with 5 μg of each of certain mixtures of (*Z*)-9-alkenes, the striking activity became as high as that of 10 μg of (*Z*)-9-tricosene. This might be a synergism effect. The mixtures of *n*-alkanes, as well as the mono- and dimethylalkanes, showed moderate activity. When (*Z*)-9-tricosene was added to these materials no increase in male activity was observed.

Key Words—Housefly, *Musca domestica*, Diptera, Muscidae, sex pheromone, (*Z*)-9-tricosene, (*Z*)-9-alkenes, *n*-alkanes, monomethylalkanes, dimethylalkanes, jojoba wax.

INTRODUCTION

(*Z*)-9-Tricosene, also known as muscalure, is the main active component of the sex pheromone of the female housefly (*Musca domestica* L.) (Carlson et al., 1971). It has been demonstrated, however, that the total wash of the cuticular surface from the female (but not from the male) is 7–10 times more active towards the male than is (*Z*)-9-tricosene alone, and the hydrocarbon fraction is four to nine times more active than muscalure (Uebel et al., 1976). These findings are in contrast to previous work by Rogoff et al. (1973), who could not find higher activity of female homogenate as compared to (*Z*)-9-tricosene.

Uebel et al. (1976) showed that the cuticular hydrocarbons are composed of several groups of compounds. Further work by Nelson et al. (1981) revealed the complexity of the cuticular hydrocarbons washed from both females and males; three major groups of long-chain hydrocarbons were present: (1) (*Z*)-9-alkenes, (2) *n*-alkanes, and (3) mono- and dimethylalkanes.

Comparison between female and male 4-day-old flies showed that most of the components appear in both sexes, but in different ratios (Table 1). The (*Z*)-9-alkenes are minor components in the female but major ones in the male; (*Z*)-9-tricosene constitutes only 4.3% of the total mixture in the female [ca. 30% of the (*Z*)-9-alkenes] and is completely missing in male washings (Table 2). Silhacek et al. (1972b) showed that while there is no change in the contents of cuticular hydrocarbons in the male, the amounts of C₂₃ and C₂₉ hydrocarbons in the female increase at the expense of (*Z*)-9-heptacosene during the posteclosion period and before mating. Dillwith et al. (1983) showed that (*Z*)-9-tricosene is biosynthesized up to the age of 6 days (by which time the female ovaries have matured) mainly at the expense of (*Z*)-9-heptacosene and to some degree of (*Z*)-9-nonacosene.

Studies on the activity of different groups of synthetic chemicals have been conducted previously. A 7:3 mixture of (*Z*)-9-tricosene and (*Z*)-9-heneicosene

TABLE 1. COMPOSITION OF THREE MAIN GROUPS OF CUTICULAR HYDROCARBONS WASHED FROM HOUSEFLY^a

Component	Composition (%)	
	Male	Female
(<i>Z</i>)-9-Alkenes	60	14
<i>n</i> -Alkanes	36	50
Methylalkanes	4	36

^aTaken from Nelson et al. (1981).

TABLE 2. RELATIVE COMPOSITION OF (Z)-9-ALKENES IN MALE AND FEMALE CUTICULAR HYDROCARBONS

Component	Male		Female	
	Composition (%) obtained from Nelson ^a	Relative composition (%)	Composition (%) obtained from Nelson ^a	Relative composition (%)
(Z)-9-C ₂₁			tr	
(Z)-9-C ₂₃			4.3	30.5
(Z)-9-C ₂₆	0.2	0.3		
(Z)-9-C ₂₇	51.0	85.6	2.8	19.9
(Z)-9-C ₂₈	0.5	0.8		
(Z)-9-C ₂₉	6.8	11.4	2.0	14.2
(Z)-9-C ₃₁	0.4	0.7	3.6	25.5
(Z)-9-C ₃₃	0.7	1.2	1.4	9.9
Total	59.6	100.0	14.1	100.0

^aTaken from Nelson et al. (1981).

was claimed to cause higher biological activity than did pure (Z)-9-tricosene (Mansingh et al., 1972), although this is unconfirmed (Carlson et al., 1974; Richter, 1974; Richter et al., 1976). Both lower and higher homologs (C₁₉-C₂₉) as well as different isomers, as single components, showed lower and higher activity (Carlson et al., 1971, 1974; Mansingh et al., 1972; Rogoff et al., 1973; Uebel et al., 1976). Mixtures of (Z)-9-alkenes (C₁₈-C₂₅) also showed some activity (Richter et al., 1976). Preliminary studies on mixtures of (Z)-9-alkenes, prepared from jojoba wax (Shani, 1979), showed activity comparable to muscalure (Carlson, personal communication). Mixtures of *n*-alkanes did not show any enhanced activity even when added to (Z)-9-tricosene (Silhacek et al., 1972a; Uebel et al., 1976). Several mono- and dimethylalkanes were also found to be active (Uebel et al., 1976; Rogoff et al., 1980; Adams and Holt, 1987). Two oxygenated products [(Z)-9,10-epoxytricosene and (Z)-14-tricosene-10-one] were claimed to be active (Uebel et al., 1978), but it was later shown that these chemicals were not active in odor tests (Adams et al., 1984). In a recent paper it was reported that the nonhydrocarbon fraction increased copulatory attempts, but not mating strikes (Adams and Holt, 1987).

The biological importance of the various components of the female cuticular hydrocarbons is not fully known, and it would be tedious to evaluate each component singly for its activity. In addition, the mixture of components, and not a single component, is often the active substance. Synergism effect could be another factor in the activity of mixtures. We thus sought a readily available

source that could supply several components in one preparation in order to study the activity of such mixtures. The source is found in jojoba wax. Based on these considerations and early encouraging results from jojoba wax preparations (see above), we widened our studies.

We report here on the synthesis of (*Z*)-9-alkenes and their hydrogenated *n*-alkanes from jojoba wax (I) (composition, Table 3), i.e., two groups of the chemicals typically found in cuticular washings, and of two methylalkanes and two dimethylalkanes. We then studied the biological activity of all the above-mentioned mixtures.

METHODS AND MATERIALS

The synthesis of the (*Z*)-9-alkenes from jojoba wax followed our previously published procedure (Shani, 1979). The fractions collected in the distillation step were distilled in a wide range of temperatures in order to keep the

TABLE 3. COMPOSITION OF JOJOBA WAX AND ITS COMPONENTS

Jojoba wax ^a		Acid and alcohol components of jojoba wax ^a		
Chain length	Composition (%)	Chain length	Composition (%)	
			Acid	Alcohol
32	tr	12:0	tr	
34	0.2	14:0	tr	tr
35	0.1	15:0	tr	
36	2.0	16:0	1.6	0.2
37	0.2	16:1	0.4	
<u>38^b</u>	<u>11.0</u>	17:0	0.1	
39	0.3	17:1		0.1
40	<u>33.6</u>	18:0	0.1	0.2
41	0.1	(<i>n</i> - 9) <u>18:1</u>	<u>13.1</u>	0.9
42	<u>42.8</u>	(<i>n</i> - 7) 18:1	<u>0.9</u>	
44	<u>8.2</u>	18:2	0.3	tr
46	1.3	18:3	0.2	
48	0.2	19:0	tr	
		20:0	0.1	0.3
		<u>20:1</u>	<u>70:8</u>	<u>51.9</u>
		<u>22:0</u>	<u>0.2</u>	<u>1.0</u>
		<u>22:1</u>	<u>11.2</u>	<u>38.1</u>
		24:0	0.1	0.2
		<u>24:1</u>	0.9	<u>6.1</u>

^a Taken from Graille et al. (1986).

^b Underlining of values indicates the major components.

mixture of olefins. The fractions, the composition of which is given in Tables 4-6 and most of which were used in the biological studies, were distilled at the following temperatures:

(Z)-9-Alkene derived from	Elongation unit	(Z)-9-Alkene mixture No.	Boiling point	
			°C at	mm Hg
Reduction of jojoba wax	C ₃	II	105-113	10 ⁻³
	C ₅	III	140-160	8 × 10 ⁻²
	C ₇	IV	130-190	10 ⁻³
	C ₉	V	160-200	7 × 10 ⁻²
Jojobyl alcohol	C ₃	VI	168-172	5 × 10 ⁻²
	C ₅	VII	120-180	5 × 10 ⁻²
	C ₇	VIII	160-182	7 × 10 ⁻²
	C ₉	IX	160-210	7 × 10 ⁻²
Reduced jojoboic acid	C ₃	X	120-125	10 ⁻³
	C ₅	XI	128-132	10 ⁻³
	C ₇	XII	140-190	10 ⁻³
	C ₉	XIII	160-210	7 × 10 ⁻²

Hydrogenation of the (Z)-9-alkenes (0.25-0.45 g) (II-XIII) in 30 ml ethanol and 3 ml petroleum ether together with 10-20 mg of 5% Pd/C was conducted at atmospheric pressure to yield 90-96% of saturated hydrocarbons (XIV-XXII). All NMR spectra showed two peaks at δ 0.75-0.85 and 1.1-1.3.

The mono- and dimethylalkanes synthesis followed the same procedure as for the elongation of jojoba derivatives (Shani, 1979).

GLC analyses were performed with a Packard model 417 chromatograph

TABLE 4. RELATIVE COMPOSITION OF (Z)-9-ALKENES DERIVED FROM REDUCED JOJOBA WAX^a

Product of	No. of carbon atoms						
	21	23	25	27	29	31	33
II, C ₃ elongation	8	49	29	4			
III, C ₅ elongation		7	52	28	3		
IV, C ₇ elongation			6	60	28	3	
V, C ₉ elongation				6	50	31	4

^aValues in the table are percentages.

TABLE 5. RELATIVE COMPOSITION OF (Z)-9-ALKENES DERIVED FROM JOJOYL ALCOHOL^a

Product of	No. of carbon atoms						
	21	23	25	27	29	31	33
VI, C ₃ elongation	1	30	41	13			
VII, C ₅ elongation		2	42	40	8		
VIII, C ₇ elongation			2	49	42	8	
IX, C ₉ elongation				2	46	42	3

^aValues in the table are percentages.

on a 2-m × 2-mm ID glass column, 10% SP 2300 on acid-washed Chromosorb W at 190°C. GC-MS measurements were performed on a Varian MAT-112 instrument.

Insects. The houseflies were collected during the summer of 1984 from a cowshed at Kibbutz Hatzerim (near Beer-Sheva). They were reared in the laboratory in net cages 20 × 20 × 30 cm and supplied with water and a sugar solution on cotton wool. The temperature was 27 ± 1°C, the relative humidity 70–80%, and the photophase–scotophase was 14:10 light–dark. On the fifth day, the water was replaced by milk on cotton wool where the females later laid their eggs. Each day the cotton wool was replaced, and the “old” cotton wool was placed in a bowl of milk powder with wheat bran in which the larvae were reared. Within an hour after emerging, adult flies were cooled to 5°C and then sexed. Males and females were kept separately in 1-liter beakers (70–100 flies each) containing sugar and water and covered with filter paper.

Bioassay. On the day the flies were tested, the males were anesthetized with diethyl ether and put in fresh plastic Petri dishes (9 cm in diameter). Five males were placed in each dish, and 10 dishes were assayed or tested in each experiment (total of 50 males). Each dish was covered with a plastic cover

TABLE 6. RELATIVE COMPOSITION OF (Z)-9-ALKENES DERIVED FROM REDUCED JOJOBOIC ACID^a

Product of	No. of carbon atoms						
	21	23	25	27	29	31	33
X, C ₃ elongation	16	49	25	3			
XI, C ₅ elongation		17	58	20	2		
XII, C ₇ elongation			20	57	14	2	
XIII, C ₉ elongation				19	58	13	2

^aValues in the table are percentages.

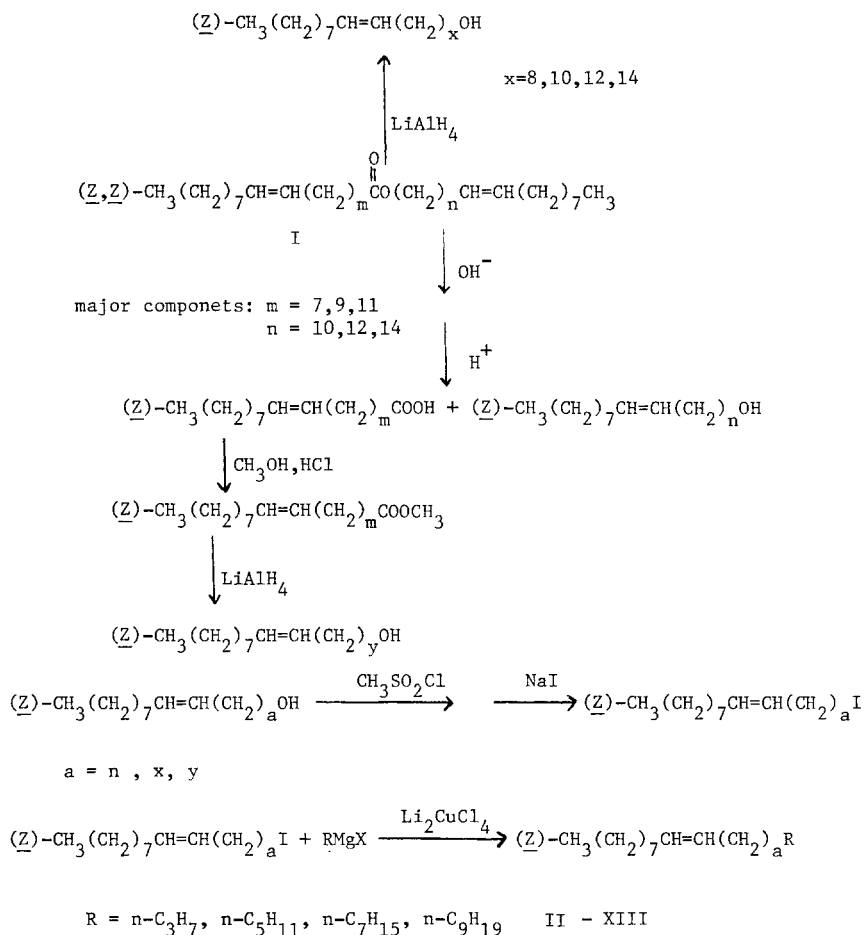
having a hole in its center through which the female model treated with the test chemical was introduced into the dish. The hole was then plugged with cotton wool. The males recovered from anesthesia within an hour, after which the experiment commenced. The female models (1–2 days old), previously frozen at -5°C , were then washed with petroleum ether, air dried in the sun for 5 min, and finally treated with the test chemicals. The test chemicals, prepared in petroleum ether solution, were applied to the back of the female and left there for 10–15 min so that the solvent could evaporate. One treated dead female was then introduced to each dish. The test, which was then started immediately, consisted of counting the number of mating strikes for 15 min (with the exception of the preliminary study designed to evaluate the proper age of the males and females, which lasted for 30 min). The mating strike behavior was taken to step 4 in the following sequence of male behavior towards live or dead unwashed female houseflies or towards dead washed flies treated with the chemicals to be tested: (1) apathy—completely ignoring the female; (2) low frequency of approaching the female—standing next to her for a few seconds only; (3) excited jumping next to the female; (4) Climbing on the female's back and initiating courtship; and (5) approaching the female for a second and immediately running away, followed 5 min later by jumping, running, and erratic behavior (this occurred when too high concentrations of certain chemicals were tested).

Before each experiment, the activity of a sample of virgin males (6 to 7 days old) was checked with dead unwashed females (6 to 7 days old). Only a population found to be active was used in the experiments. If the tested males did not show any activity towards the test materials, then the washing from the female was reapplied to her to verify its activity. These double checks proved both that the males were sexually active and that the tested chemicals caused either little or no activity.

For each experiment, fresh males and females were used in new Petri dishes. All the tests were run between 9 AM and 1 PM. In some cases the males became "contaminated" with the tested chemicals and sometimes homosexual activity was observed. Only experiments in which the homosexual activity was nil or very low are reported here.

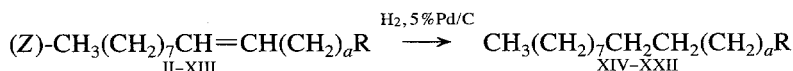
RESULTS AND DISCUSSION

We have already shown that jojoba wax (I) is a suitable source of long-chain (*Z*)-9-alkenes (Shani, 1979), since it is a mixture of long-chain esters (Miwa, 1971; Graille et al., 1986) composed mainly of C_{18} – C_{24} acids and alcohols having a *Z* double bond in the appropriate position. The synthesis is based on elongation of the corresponding jojoba components by short alkyl chains (C_3 – C_9), as described in Scheme 1. Because of the difference in com-



SCHEME 1.

position of the esters, as shown in Table 3, three different mixtures are obtained by reduction or hydrolysis (as shown in Scheme 1) from one elongation procedure and a total of 12 mixtures by applying four different alkyl chains. After purification and distillation, the relative compositions of the fractions that were later studied were measured on a GLC (Tables 4-6). These fractions were later hydrogenated to the *n*-alkanes, thus having the same composition of the olefin mixtures.

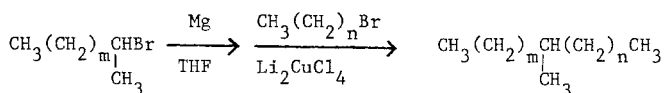


The NMR spectra showed no olefinic hydrogens in the hydrogenated products.

The mono- and dimethylalkanes were synthesized by Li_2CuCl_4 -catalyzed coupling of a Grignard reagent with the appropriate alkyl halide (Tamura and Kochi, 1974), as described in Scheme 2.

The first step in the bioassay was to determine the most active age of virgin males toward virgin females. The results are summarized in Table 7 and in Figures 1 and 2, where the curves represent a specific age (in days) of females and males, respectively. It is clear that between the ages of 5 and 8 days, the virgin males are active towards 5- to 8-day-old virgin females (killed immediately before the experiment). This finding is not in agreement with that of Silhacek et al. (1972b), who claimed that maximum male activity towards female washings was at the age of 2 days. Mansingh et al. (1972) and Adams et al. (1984) found the age of 3 days suitable for these experiments. Richter et al.

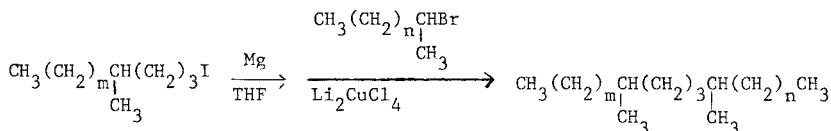
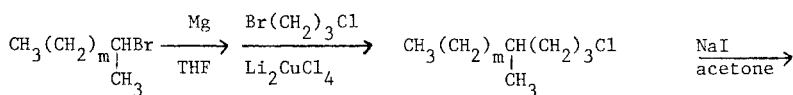
Monomethylalkanes



XXIII $m = 9, n = 17$: 11-Methylnonacosane

XXIV $m = 3, n = 21$: 5-Methylheptacosane

Dimethylalkanes



XXV $m = 5, n = 15$: 7,11-dimethylheptacosane

XXVI $m = 3, n = 17$: 5,9-dimethylheptacosane

SCHEME 2.

TABLE 7. AVERAGE NUMBER OF MATING STRIKES BY MALES IN 10 REPLICATES

Male age (days)	Female age (days) ^a						
	4	5	6	7	8	9	10
4	0.4	0.6	0.9	1.8	0.6	0.4	0.0
5	0.5	1.5	3.1	4.0 ± 1.15	1.0	0.5	0.0
6	1.1	3.5	7.8	8.5 ± 1.08	4.0	1.6	0.1
7	1.0	6.0 ± 1.16	9.7 ± 1.82	12.6 ± 1.74	6.3 ± 1.34	1.9	0.1
8	0.6	0.2	6.3	5.3 ± 0.95	2.3	1.0	0.1
9	0.3	0.9	2.3	2.9	1.0	0.1	0.0
10	0.1	0.3	0.5	0.7	0.4	0.0	0.0

^a Values are the mean numbers (± SE) per dish in which the behavior of five virgin males of different ages was examined for 30 min towards one dead unwashed virgin female at the age indicated.

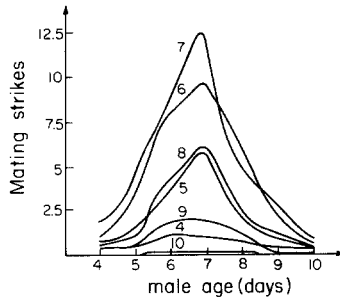


FIG. 1. Average number of mating strikes by males towards females as function of male age (in days). Each curve represents female with the specific age (days). Based on Table 7.

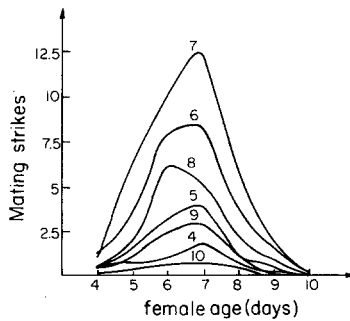


FIG. 2. Average number of mating strikes by males towards females as function of female age (in days). Each curve represents male with the specific age (days). Based on Table 7.

(1976) studied the activity of 5- to 10-day-old males. The maximum amount of (*Z*)-9-tricosene on the female was obtained at the age of 6 days (starting at the age of 4 days) according to Dillwith et al. (1983). Rogoff et al. (1973) found high pheromone production during the age period of 4–8 days; Adams et al. (1984) studied the activity from day 1 until day 7; and Adler et al. (1984) measured the cuticular lipids of the female at the age of 10 days. The thorough study of Nelson et al. (1981) was performed on 4-day-old males and females. As our findings cover broad age ranges for both males and females, we may conclude that the best age for study of both sexual activity of males and sex pheromone composition of females is 6–7 days, i.e., the age selected for our studies. On the other hand, because no activity was found towards 1- to 2-day-old females, we selected these young females as the carriers for the chemicals to be tested, after washing them with petroleum ether.

An additional reason for selecting females as models and not applying the pseudofly test (knots) stemmed from the possible effect of vision on the sexual behavior of the males (Richter et al., 1976). After completion of our studies, we found that Adams and Holt (1987), in their very recent study, could detect a lower degree of reaction of males, as compared with females, towards knots in most experiments. No significant difference was detected between the reaction of males toward male and female models and towards 3-day-old female controls.

Each experiment was preceded by confirming the sexual activity of a male sample of a population towards a freshly killed female and ensuring that, after washing the female with petroleum ether, the typical activity of the male disappeared. Reapplication of this washing to the female's back stimulated resumption of the activity of the males.

The next step was to determine the optimum amount of the chemicals to be tested. There are conflicting reports on the quantity needed per housefly. Silhacek et al. (1972a), Carlson et al. (1974), and Morgan et al. (1974) applied milligram amounts of tricosene and other components, while Rogoff et al. (1980), Nelson et al. (1981), Dillwith et al. (1983), and Adams and Holt (1987) found and/or applied microgram quantities. Uebel et al. (1976, 1978) and Richter et al. (1976) applied 100–200 μg of the tested chemicals, while Adler et al. (1984) found only nanogram quantities of tricosene per female. It is possible that this small amount was a function of the very late age (10 days) of the flies tested (see above). Increasing the amount of applied tricosene from 1 to 100 μg per female showed that the maximum activity of males occurred at 10–25 μg of (*Z*)-9-tricosene, while at 1–5 μg no activity was found, and at 50–100 μg the males behaved erratically (level 5 of our grading, see Methods and Materials). Therefore, we measured the activity with 10–25 μg of tested materials and compared it to that obtained with 10 μg of tricosene. These quantities are in the range of the physiological composition as discussed recently by Adams

TABLE 8. AVERAGE NUMBER OF MATING STRIKES BY MALES IN 10 REPLICATES WITH (Z)-9-ALKENE MIXTURES^a OBTAINED FROM REDUCTION AND HYDROLYSIS OF JOJOBA WAX, AS COMPARED WITH TRICOSENE^b

Elongation	Reduced		Alcohol		Acid	
		jojoba wax				
C ₃	II	5.3a	VI	1.9bcde	X	2.6b
C ₅	III	2.5b	VII	1.6bcd	XI	c
C ₇	IV	1.2de	VIII	c	XII	0.6c
C ₉	V	1.3cd	IX	1.8bcd	XIII	2.3bc
Tricosene		6.9a		6.9a		6.9a

^aAll studies were performed with 10 μ g of tested mixture, except for 5 μ g in X.

^bThe mean numbers are given per dish (five males) for a 15-min test period. Those means followed by the same letter are not significantly different from each other at $P = 0.05$ (Student's t test).

^cNot studied.

and Holt (1987). They applied 4 μ g of tricosene, which was found to be about 4.3% of the total wash and about 30% of the (Z)-9-alkenes fraction (see Tables 1 and 2); thus 10 μ g of our (Z)-9-alkenes (Table 8) and 5 μ g of the (Z)-9-alkenes-tricosene mixtures (Table 9) are consistent with the quantities used by Adams and Holt (1987). However, since Adams and Holt applied 7 μ g of the methylalkane fraction (about 36% of total wash, Table 1), it could be that the amounts of 10–15 μ g each of our single components are too high (see below).

TABLE 9. AVERAGE NUMBER OF MATING STRIKES OF MALES IN 10 REPLICATES WITH MIXTURES OF (Z)-9-ALKENES DERIVED FROM JOJOBA WAX AND TRICOSENE^a

Entry	Tested mixtures (μ g)		Average No. of mating strikes	Composition (%) of tested mixtures						
	(Z)-9-alkene	Tricosene		C ₂₁	C ₂₃	C ₂₅	C ₂₇	C ₂₉	C ₃₁	C ₃₃
1	VII	5	5	2.3d		51.0	21.0	20.0	4.0	
2	II	5	10	3.4cd	2.7	83.3	9.7	1.3		
3	IV	5	5	4.3bc		50.0	3.0	30.0	14.0	1.5
4	III	5	5	6.1ab		53.5	26.0	14.0	1.5	
5	XII	5	5	6.0a		50.0	10.0	28.5	7.0	1.0
6	V	5	5	6.0a		50.0		3.0	25.0	15.5
7	II	5	5	5.9ab	4.0	74.5	14.5	2.0		
8		10	6.9a			100				

^aThe mean numbers are given per dish (five males) for a 15-min test period. Those means followed by the same letter are not significantly different from each other at $P = 0.05$ (Student's t test).

We found that 10 μg of tricosene caused high activity of the males, up to 6.9 ± 1.3 mating strikes per dish of five males for 15 min. Qualitative comparison (Table 7 and Figures 1 and 2) between the behavior of the males toward dead unwashed females (which showed 12.6 ± 1.74 mating strikes per dish of five males for 30 min) and toward tricosene clearly demonstrates that this component of the (*Z*)-9-alkene fraction is the main component responsible for mating striking activity. Other components are probably involved in the complete sequence (Adams and Holt, 1987).

The tested chemicals were divided into three groups according to the main categories of hydrocarbons found on the housefly cuticle. The synthetic (*Z*)-9-alkenes mixtures tested represent 10 different compositions (out of the possible 12), and their effect on the sexual activity of the male is summarized in Table 8. Except for mixture II, all showed low activity as compared with tricosene. Comparison of the relative composition of (*Z*)-9-alkenes (Table 2) and the composition of the synthetic alkenes (Tables 4–6) shows that none match exactly. However, when mixtures of some of these synthetic chemicals with tricosene were applied, a significant increase in activity was found (Table 9). Still, some inconsistency was observed in that similar compositions did not always cause the same activity (entries 1 and 4 or 2 and 7 in Table 9). It could be that the minor components, which were not taken into account here, are partly responsible for the different activities of the various mixtures. On the other hand, mixtures that are different in composition (Table 9, entries 4 and 6 or 5 and 6) caused similar activity in males. These results do not point to a simple answer with regard to the effect of (*Z*)-9-alkenes on the male mating strike activity caused by tricosene. One possible explanation for these and previous inconsistent results could be a synergism effect of certain (*Z*)-9-alkenes in the synthetic mixtures, which resemble or even are identical to the natural cuticular hydrocarbons washed from the female.

The effect of *n*-alkanes on males was studied by Silhacek et al. (1972a), who found that a mixture of 10 mg of even-numbered chains from C_{20} to C_{36} showed low activity and a mixture of C_{25} – C_{30} was inactive. Uebel et al. (1976) found that the saturated hydrocarbons alone, separated from the female cuticular washing, showed no activity, but a mixture of 65% of saturated and 35% of unsaturated hydrocarbon fractions was 8–10 times more active than muscalure. They also found that when a mixture of tricosene and saturated C_{27} , C_{28} , C_{29} , and C_{30} straight chains was studied, no increase in mating strike was found as compared with tricosene. Our results show that the low to medium activity (1.4–4.5 mating strikes per dish, five males), as compared with tricosene (6.9 mating strikes per dish), fluctuated when the *n*-alkanes were studied alone or as mixtures with tricosene. In this case we could not see the enhancing effect of tricosene as appeared with the (*Z*)-9-alkenes (see above). These findings are in accordance with those of Uebel et al. (1976). It is interesting to note that

50% of the female cuticular hydrocarbons (see Table 1) are *n*-alkanes. It is likely that the hydrocarbons serve as the "solvent" in which the active components are retained to lengthen their active period.

The methylalkanes comprise a rich mixture of mono- and dimethylalkanes, substituting many sites along the chains and also differing in length (Nelson et al., 1981). Their content is more than a third of the female cuticular hydrocarbons (Table 1), but the amount of each component is probably very small. The major groups are derived from C₂₇, C₂₉, and C₃₁ (8.7, 17.4 and 5.7%, respectively). Therefore, any selection of specific methylalkanes to be tested for biological activity is arbitrary. In spite of this, Uebel et al. (1976) and Rogoff et al. (1980) could show enhanced activity caused by several methylalkanes, such as 11-methylheptacosane, 13-methylheptacosane, 11-methylnonacosane, 13-methylnonacosane and 4,8-dimethylheptacosane, when mixed with tricosene, although the individual methylalkanes were significantly less active than tricosene. We prepared 11-methylnonacosane (XXIII), 5-methylheptacosane (XXIV), 7,11-dimethylheptacosane (XXV), and 5,9-dimethylheptacosane (XXVI). These components are present in the larger fractions of the methylalkanes (Nelson et al., 1981). The intermediate activity of these compounds (2.9–3.6 average number of mating strikes per dish, five males) is slightly increased when applied as a mixture with tricosene but is not significantly different from their activity as single components. We used ratios of 2:1 methylalkane-tricosene; in the case of 11-methylnonacosane (XXIII), however, increasing the ratio of XXIII slightly enhanced the activity from 2.4 to 3.3 mating strikes per dish. As mentioned above, it could be that the amounts we applied were too high with regard to physiological quantities, as determined in another strain (Adams and Holt, 1987). As long as there is no quantitative information on the methylalkane fraction of the cuticle, studies on the individual components are less likely to succeed.

The results of our study confirm that (*Z*)-9-tricosene is the main active component of the sex pheromone produced by the female housefly, which initiates mating strike activity in the male. The (*Z*)-9-alkenes associated in the cuticular lipids probably act as synergists to muscalure. Both *n*-alkanes and methylalkanes, comprising 86% of total cuticular female hydrocarbons, show low to medium activity, although the addition of (*Z*)-9-tricosene does not enhance it. This leaves open the question whether these components have any biological importance in the sex pheromone activity of the housefly. They probably serve as the matrix from which the sex pheromone [(*Z*)-9-tricosene and the oxygenated components] is slowly released and kept from chemical degradation. Minor synergism activity could be attributed to some methylalkanes.

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KAIROMONAL ACTIVITIES OF 2-ACYLCYCLOHEXANE-1,3 DIONES PRODUCED BY *Ephestia kuehniella* ZELLER IN ELICITING SEARCHING BEHAVIOR BY THE PARASITOID *Bracon hebetor* (Say)

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Abstract—2-Acylcyclohexane-1,3-diones produced in the mandibular glands of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) fifth instars acted as arrestment and host-trail following kairomones for the parasitoid, *Bracon hebetor* (Say) (Hymenoptera: Braconidae). The behavioral response of the parasitoids to the kairomone varied with kairomone concentration and distribution. However, only small differences in activity were noted for the different mandibular gland components. *B. hebetor* females that encountered filter paper patches impregnated with the kairomone exhibited antennation and probing behavior. Females followed trails formed with 2-[(Z,E)-12,14-hexadecadienoyl]cyclohexane-1,3-dione in the same manner exhibited with host-made trails. At concentrations of 1.0 $\mu\text{g}/\text{mm}$ and above, trail following was interrupted by frequent probing.

Key Words—*Bracon*, *Ephestia*, Lepidoptera, Pyralidae, Hymenoptera, Braconidae, kairomone, host selection, parasitoid, foraging, ketones, 2-acylcyclohexane-1,3-diones.

INTRODUCTION

Bracon hebetor (Say) is a gregarious larval ectoparasitoid of stored-product moths in the family Pyralidae (Ulyett, 1945; Hagstrum and Smittle, 1978; Krombein *et al.*, 1979). Host larvae from genera such as *Plodia* and *Ephestia* live gregariously in stored grains, cereals, nuts, and dried fruit, often building to very high densities in warehouses. *B. hebetor* prefers late-instar hosts after they move to the surface to wander in search of a pupation site (Benson, 1973). Murr (1930) was the first to present evidence that *B. hebetor* females are arrested by and search the substrate contacted by hosts. Once located, the female injects a small quantity of venom into the host which induces complete paralysis in 15 min (Piek *et al.*, 1978). After paralysis, females mount and externally examine the host. If the host is found to be acceptable, the female oviposits, usually placing the clutch of eggs on the side of the host in contact with the substrate.

The presence of host kairomones along with the high reproductive rate and short generation time of *B. hebetor* offer opportunities for manipulating this parasitoid in the management of stored-product larvae (Hagstrum and Smittle, 1978). Here we report the source and identification of arrestment and trail following kairomones for *B. hebetor* produced by *Ephestia kuehniella* Zeller fifth instars.

METHODS AND MATERIALS

E. kuehniella larvae were reared on cornmeal in 2 liter plastic containers maintained at 27°C, 70% relative humidity, and a 16:8 light-dark photoperiod. *B. hebetor* adults were maintained in 50-ml glass culture tubes and fed a 50% honey solution daily. Hosts used in culture maintenance were parasitized in 100 × 15-mm Petri dishes. Both the *E. kuehniella* and *B. hebetor* cultures had been maintained in the laboratory for approximately three years. Parasitized hosts were held at 27°C, 70% relative humidity, and a 16:8 light-dark photoperiod. Parasitoid development from egg to adult emergence took ca. 13 days.

All parasitoids used in the study were 6–7 days old and were exposed to hosts two days prior to use in an experiment. No parasitoid was used more than once during the study. Bioassays were conducted in 100 × 15-mm Petri dishes lined with Whatman No. 1 filter paper. A 1-cm circle was drawn with a pencil in the middle of the filter paper to which materials for bioassay were applied. The area within the 1-cm circle is hereafter referred to as a patch. No more than two females were exposed to any patch. All experiments were conducted during daylight hours at 25°C and ca. 40% relative humidity.

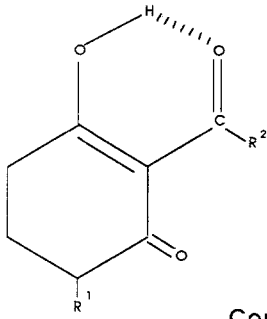
In previous studies it was found that several stored-product pyralid larvae secrete material from their mandibular glands while feeding and moving (Cor-

bet, 1971; Mudd and Corbet, 1973, 1982; Mossadegh, 1978; Mudd, 1983). Thus, we initially examined the behavioral response of *B. hebetor* females to an ether extract of the mandibular glands from two *E. kuehniella* fifth instars. The two glands from each larva were removed with forceps under Ringer's saline, and placed in 200 μ l of ether. The extract was then uniformly applied to the 1-cm patch of the Petri dish bioassay chamber. Patches with only ether applied served as negative controls. After evaporation of the ether, *B. hebetor* females were introduced individually into the Petri dish and observed. A positive control consisted of restricting four *E. kuehniella* fifth instars for 5 hr to a patch by a 1-cm circular plastic lid. The larvae and any frass pellets were removed from the patch before individually introducing females for observation.

The response of *B. hebetor* females to these treatments was based upon the number of wasps that positively responded to the patch and the duration of the patch visit. A positive response was characterized by antennation of the substrate, a reduced walking speed, and probing with the ovipositor. A patch visit was designated as beginning when a female first entered the patch and ending when a female left the patch for more than 30 sec. The 30-sec value for having left a patch was adopted because, on occasion, a female would walk beyond the patch border only to return quickly and resume searching. Although arbitrary, the 30-sec value was a reliable indicator of a patch visit. Any time spent outside of the patch border was not included in the time recorded for a patch visit. *B. hebetor* walking speed outside of and within the patch was estimated by measuring the distance traveled by 20 females in the last 5 sec before encountering the patch and the first 5 sec after entering the patch. The distances traveled during these periods were recorded by tracing the wasp's path on the cover of the Petri dish bioassay chamber with a felt pen. Such tracing did not appear to disturb the parasitoids. The distance traveled was calculated using a map measurer.

The response of *B. hebetor* to the previously isolated and characterized 2-acylcyclohexane-1,3-diones and 4-hydroxylated compounds from *E. kuehniella* mandibular glands (Mudd, 1983; Mudd *et al.*, 1984) (Figure 1) was then examined. The compounds listed in Figure 1 were obtained by the methods outlined by Mudd (1983). Fractions IA and IB chromatographed as single components (Mudd *et al.*, 1984) and were tested as such. Doses of 0.01, 0.1, 1.0, and 10.0 μ g in ether were applied uniformly to the 1-cm patch as described previously. After evaporation of the ether, *B. hebetor* females were individually introduced into the Petri dish where the response to and duration of the patch visit was recorded. As with testing of the mandibular gland extracts, a positive response was characterized by a reduced walking speed, antennation, and probing of the substrate.

Testing of trail-following activity by *B. hebetor* was performed by uni-



Component	R ¹	R ²
1	H	
2	H	
3	H	
4	H	
5	H	
6	H	
7	H	
8	OH	
9	OH	
10	OH	
11	OH	
12	OH	
13	OH	
14	OH	

FIG. 1. Compounds identified from mandibular gland secretions of *E. kuehniella* that were tested with *B. hebetor*. Components 1-14 are listed with the appropriate side chain, and the unseparated components 2-4 and 9-11 designated as fractions IA and IB, respectively.

formly applying an ether solution of component 1 (see Figure 1) in a 3-cm-diameter circle on Whatman No. 1 filter paper with a syringe at 0.01, 0.1, and 1.0 $\mu\text{g}/\text{mm}$. A negative control consisted of an ether-only trail, and a positive control consisted of allowing two wandering stage *E. kuehniella* fifth instars to crawl for two rotations around the inside wall of a 3-cm Petri dish lid. After evaporation of the ether or removal of the *E. kuehniella* larvae, *B. hebetor* females were introduced individually into the Petri dish and observed. A positive response was recorded if, upon contact with the trail, females followed the compound or host track for a minimum of one rotation. Females were removed from the assay chamber after departing from the trail or if one rotation was completed.

RESULTS

All *B. hebetor* females responded to patches with *E. kuehniella* mandibular gland extract or on which *E. kuehniella* fifth instars had crawled, but no females responded to the ether control (Table 1). Before encountering the patch, *B. hebetor* females generally walked in a straight path across the Petri dish floor. Movement immediately prior to and directly after a patch encounter showed no directed movement toward the patch. However, when the edge of a mandibular gland extract or host-contaminated patch was encountered, females began to antennate the patch edge.

After entering the patch, the movement of females was characterized as a combination of orthokinetic movement within the patch combined with a klinotactic response to the patch border. While searching within the patch, females reduced their walking speed as they antennated, probed, and turned. Outside of the patch mean walking speed was 1.38 cm/5 sec \pm 0.59 SD ($N = 20$), while

TABLE 1. PERCENTAGE OF *B. hebetor* FEMALES RESPONDING TO AND MEAN TIME SPENT IN PATCHES MADE BY *E. kuehniella* FIFTH INSTARS AND MANDIBULAR GLAND EXTRACT

Patch type	Females tested (N)	Positive response (%) ^a	Mean patch duration (sec \pm SE) ^b
Mandibular glands	40	100	96.2 \pm 17.4*
<i>E. kuehniella</i> fifth instars	52	98	117.1 \pm 20.9*
Ether (control)	25	0	1.4 \pm 0.2†

^aResponse was characterized by a female exhibiting arrestment, antennation, and probing behavior (see Methods and Materials).

^b $F(2, 115) = 48.6, P < 0.0001$; means followed by the same symbol are not significantly different (SNK multiple comparison procedure, $\alpha = 0.01$).

within the patch mean walking speed was $0.37 \text{ cm/5 sec} \pm 0.24 \text{ SD}$ ($N = 20$). When females encountered the patch border, they usually turned sharply back toward the patch while continuing to antennate. Mean examination time for females in patches treated with mandibular gland extract or on which host larvae had crawled were similar, but females spent very little time in patches treated with ether only (Table 1).

The amounts of time *B. hebetor* spent in patches treated with increasing concentrations of the isolated 2-acylcyclohexane-1,3-diones, 4-hydroxy compounds, and ether control are presented in Table 2. At concentrations of 0.01 and 0.1 μg of material per patch, there was a tendency for females to spend more time in patches treated with components 1 and 8. However, at higher concentrations females responded similarly to all the components. There was usually little response to patches treated with 0.01 μg of the isolated mandibular gland components. The only exception to this was a slight antennation response to components 1 and 8. Females continuously antennated patches treated with 0.1 μg of all the components and probed patches treated with components 1 and 8. Females were arrested, antennated, and probed patches treated with 1 or 10 μg of all the components. At these higher concentrations, it was common for females to spend 5–10 min antennating and probing the substrate before leaving the patch (Table 2).

B. hebetor females followed trails made by application of component 1 or by *E. kuehniella* fifth instars but did not follow trails of ether only (Figure 2). The percentage of females that followed trails of component 1 for at least one

TABLE 2. MEAN DURATION (SEC) \pm SE OF PATCH VISITS BY *B. hebetor* IN RESPONSE TO *E. kuehniella* MANDIBULAR GLAND COMPONENTS AT DIFFERENT CONCENTRATIONS

Component ^a	Concentration ($\mu\text{g}/\text{patch}$)			
	0.01	0.10	1.0	10.0
1	4.5 \pm 0.6	111.7 \pm 23.8	183.1 \pm 30.2	477.9 \pm 50.0
1A	1.4 \pm 0.3	22.4 \pm 4.0	204.2 \pm 26.2	373.3 \pm 54.0
5	1.0 \pm 0.2	4.2 \pm 1.4	98.2 \pm 18.1	492.4 \pm 57.3
6	1.9 \pm 0.6	25.9 \pm 4.3	95.6 \pm 12.4	229.0 \pm 30.5
7	1.2 \pm 0.2	5.0 \pm 1.0	62.2 \pm 10.1	211.0 \pm 19.3
8	3.3 \pm 0.9	94.9 \pm 15.5	168.2 \pm 20.1	390.2 \pm 56.8
1B	1.6 \pm 0.3	9.1 \pm 1.6	146.6 \pm 23.3	336.3 \pm 36.0
12	1.5 \pm 0.3	8.2 \pm 1.3	61.0 \pm 11.2	431.5 \pm 53.7
13	1.5 \pm 0.4	4.7 \pm 0.9	70.5 \pm 10.2	281.4 \pm 34.2
14	1.2 \pm 0.1	4.4 \pm 1.3	66.4 \pm 9.1	243.1 \pm 19.0
Ether control	1.7 \pm 0.3	2.0 \pm 0.4	1.5 \pm 0.2	1.5 \pm 0.3

^aSee Figure 1. Twenty females were tested for each component concentration.

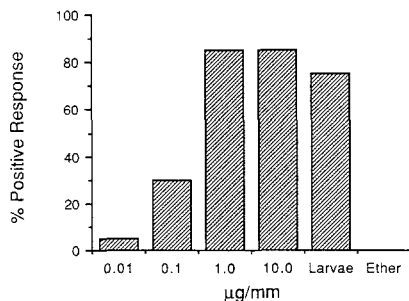


FIG. 2. Percent response of *B. hebetor* females that successfully followed for at least one rotation a circular trail made by component 1 (see Figure 1), *E. kuehniella* fifth instars, and ether.

rotation increased with kairomone concentration. Although most females partially followed trails of component 1 at a concentration of 0.1 and 0.01 $\mu\text{g}/\text{mm}$, less than half the females tested followed trails for one rotation. However, at a concentration of 1 or 10 $\mu\text{g}/\text{mm}$, the percentage of responding females did not differ from the percentage of females that responded to trails made by *E. kuehniella* larvae ($\chi^2 = 2.1$; $df = 4$; $P > 0.25$). When encountering a trail made by component 1 or by host larvae, females were initially arrested but then quickly turned in the direction of the trail and began following. Females antennated the trail continuously while following and often probed the trail at concentrations of 1.0 and 10 $\mu\text{g}/\text{mm}$. While trail following, females characteristically walked a zigzag route in which each antenna moved alternately across the trail.

DISCUSSION

In this study, we find that the 2-acylcyclohexane-1,3-diones and 4-hydroxylated compounds produced by the mandibular glands of *E. kuehniella* fifth instars act as kairomones for the larval ectoparasitoid *B. hebetor*. These compounds elicit an arrestment, antennation, and probing response when present in a patch, and a trail following response when deposited linearly. The behavioral responses observed for the isolated mandibular gland components are similar to those observed for *B. hebetor* encountering patches upon which *E. kuehniella* fifth instars had crawled or the trails they deposit while wandering in search of a pupation site (Murr, 1930; Mossadegh, 1978).

Previous study found that the same mandibular gland compounds active for *B. hebetor* elicited antennation and ovipositor thrusting behavior by the endoparasitoid *Venturia* (= *Nemeritis*) *canescens* Grav. (Mudd and Corbet, 1982; Mudd *et al.*, 1984). These compounds are also reported to serve as an

epideictic pheromone for *E. kuehniella* (Corbet, 1971). Documented examples of compounds produced from the same host having kairomonal activity for more than one parasitoid species are still not common (Kennedy, 1979; Aldrich *et al.*, 1984; Dicke *et al.*, 1984; Nordlund *et al.*, 1987). However, with host species or particular host stages often supporting multiple parasitoid species, such situations might be more common than the literature currently suggests. In addition, the hosts of generalist parasitoids may share common kairomones. *B. hebetor* and *V. canescens* both parasitize the larvae of several stored-product pyralids (Ulyett, 1945; Benson, 1973; Salt, 1976; Hagstrum and Smittle, 1978), many of which are known to produce mandibular gland secretions (Mudd and Corbet, 1973; Mossadegh, 1978). Preliminary characterization of *Ephestia cautella* mandibular gland secretions has demonstrated the presence of 2-acylcyclohexane-1,3-diones similar to those already characterized for *E. kuehniella* (Strand and Williams, unpublished results). These compounds also elicit a strong behavioral response in *B. hebetor*. Further studies of other *Ephestia*, *Plodia*, and *Cadra* species may indicate that a series of related compounds is produced in the larval mandibular glands, which have kairomonal activity for *B. hebetor* and *V. canescens*. Indeed, recent studies of the aforementioned pyralid hosts suggest that quantitative and qualitative differences exist in the 2-acylcyclohexane-1,3-diones present in frass and mandibular gland extracts (Mudd, 1983; Kuwahara *et al.*, 1983; Nemoto *et al.*, 1987).

While host-produced compounds may act as kairomones for more than one parasitoid species, their activity and the behavioral responses they elicit may differ. As with *V. canescens*, the mandibular gland components with a conjugated unsaturated side chain (components 1 and 8) elicit the greatest response at low concentration in *B. hebetor*. However, unlike *V. canescens* (Mudd *et al.*, 1984), the hydroxyl group at C-4 does not seem to alter component activity for *B. hebetor*. Also unlike *V. canescens*, high patch concentrations (1 and 10 μg) of all of the components elicited a strong behavioral response in *B. hebetor*.

Behaviorally, the responses of *B. hebetor* are consistent with its ectoparasitic habit and preference for ultimate instar hosts. Females antennate and search substrates permeated with the *E. kuehniella* kairomones, but they also follow kairomone trails. Wandering *E. kuehniella* final instar larvae apparently deposit mandibular gland material on the substrate, and *B. hebetor* is capable of following this trail. This is in contrast to *V. canescens*, which prefers earlier instar hosts that remain in the substrate and which are located by the wasp through ovipositor probing. The characteristic movement of the antennae across a trail by *B. hebetor* is similar to several descriptions of trail pheromone following behavior of ants in which chemotropotactic and klinotactic trail-following components have been reported (Matthews and Matthews, 1978; Haynes and Birch, 1985).

It should be noted that in this study, females with previous oviposition

experience on *E. kuehniella* were used. The response of inexperienced females or females that have previously encountered other pyralid hosts may differ both qualitatively and quantitatively. Further characterization of how these kairomones influence *B. hebetor* foraging and orientation is currently being investigated.

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ALDEHYDIC CONTACT POISONS AND ALARM
PHEROMONE OF THE ANT *Crematogaster*
scutellaris (Hymenoptera: Myrmicinae)
Enzyme-Mediated Production from Acetate
Precursors

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Abstract—The Dufour gland of *Crematogaster scutellaris* stores a mixture of long-chain primary acetates bearing a cross-conjugated dienone (Scheme 1, 1a-c). The poison gland contains two highly active enzymes: an acetate esterase and an alcohol oxidase. During venom emission, the constituents of both glands mix and accumulate on the sting, where the formation of the highly electrophilic aldehydes (Scheme 1, 2a-c) from their acetate precursors is initiated. Acetic acid, produced during the reaction, acts as alarm pheromone. The toxicity of the acetates (Scheme 1, 1a-c) and of the crude secretion has been assessed by topical application on *Myrmica rubra*. The acetate-containing secretion from the Dufour gland was less toxic than the enzymatically altered secretion that was rich in aldehydes. The production of acids (Scheme 1, 3a-c) was an artifact resulting from the nonenzymatic oxidation of the unstable aldehydes.

Key Words—*Crematogaster scutellaris*, ants, Hymenoptera, Formicidae, Dufour gland, contact poisons, esterase, alcohol oxidase, alarm pheromone, aldehydes.

INTRODUCTION

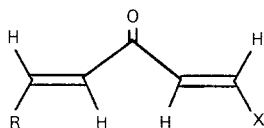
Ants of the genus *Crematogaster* are characterized by a spatulate sting, which is not a suitable injection device (Buren, 1958). In many species, the venom is emitted as a froth that accumulates on the spatulate portion and at the base of the sting, from whence it can be applied easily to the integument of attacking insects. Application is further facilitated by the fact that the abdominal tip can be pointed in nearly all directions. Indeed, *Crematogaster* species are remarkable among the ants by their ability to raise the abdomen forward and over the thorax and head. The hypertrophied Dufour gland of *Crematogaster*, which is connected to the sting, is considered to be the origin of the venom (Maschwitz, 1975; Buschinger and Maschwitz, 1984). During venom emission, an alarm pheromone is also emitted, which mediates a general excitement (i.e., frenzied alarm) in the aggregations of workers inside and outside the nest (Leuthold and Schlunegger, 1973).

In a preceding paper, we reported that the venom of *C. scutellaris* contained a series of C_{23} long-chain derivatives, characterized by the presence of a cross-conjugated dienone linked to either a primary acetate (Scheme 1, 1a-c), an aldehyde (Scheme 1, 2a-c), or a carboxylic acid function (Scheme 1, 3a-c). The aldehydes 2a-c are powerful electrophiles and could be responsible for the toxicity of the venom (Daloze et al., 1987). However, the aldehydes are highly unstable and cannot be stored as such in the Dufour gland. We report here that the Dufour gland of *C. scutellaris* only stores the acetates 1a-c and that aldehyde production is triggered at the moment of discharge of the esters to the exterior by mixing with enzymes located in the simultaneously discharged fluid of the poison gland. This enzymatic reaction also liberates acetic acid, which is responsible for the alarm reaction observed in grouped ants. The production of homologous acids 3a-c is an artifact resulting from nonenzymatic oxidation of the aldehydes 2a-c.

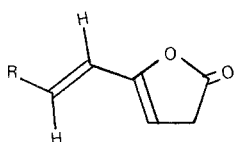
METHODS AND MATERIALS

Biological Material. Portions of dead pine logs containing ant colonies were collected in south of France near Salernes and Banyuls. These colonies could be maintained in these pieces of log for several years in the laboratory, merely by feeding them continuously with a solution of brown sugar and freshly killed cockroaches.

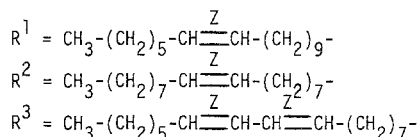
Chemical Analyses. The venom of workers was collected as described previously (Daloze et al., 1987) on bits of filter paper that were immediately dropped into *n*-hexane or CH_2Cl_2 . For the kinetic studies, the venom was collected in the same manner, but the filter papers were maintained at $19 \pm 1^\circ C$



	R	X		R	X
1a	R ¹	CH ₂ OAc	3a	R ¹	COOH
1b	R ²	CH ₂ OAc	3b	R ²	COOH
1c	R ³	CH ₂ OAc	3c	R ³	COOH
2a	R ¹	CHO	4a	R ¹	CH ₂ OH
2b	R ²	CHO	4b	R ²	CH ₂ OH
2c	R ³	CHO	4c	R ³	CH ₂ OH



	R
5a	R ¹
5b	R ²
5c	R ³



SCHEME 1.

for 0, 5, 10, or 15 min before being dipped into CH₂Cl₂. The resulting solutions were analyzed by photodensitometry using TLC plates (Merck Silicagel G F 254); eluent 1: hexane-acetone 8:2; eluent 2: CH₂Cl₂. The compounds were made visible by quenching of the UV fluorescence of silica. Absorbances were measured at 250 nm on a Shimadzu CS 930 dual-wavelength TLC scanner. Percentages given in Table 1 are mean values, obtained by averaging the values of three replications. Similar assays were performed under a nitrogen atmosphere. Enzyme specificity assays were conducted in 0.05 M phosphate buffer (pH 7.0), containing 0.1% of Tween 60 as detergent. One or 2 μg of the acetate substrate was suspended into 20 μl of the buffer solution, and two poison glands were added. After sonication for 2 min, the solution was incubated for 20 min at 20°C, then extracted twice with 5 μl of CH₂Cl₂. The organic extract was spotted on a TLC plate and analyzed for the presence of alcohol or aldehydes.

As previously reported (Daloze et al., 1987), neither the major constituents

TABLE 1. KINETICS OF PRODUCTION OF ALDEHYDES WHEN DUFOUR GLAND AND POISON GLAND WERE CRUSHED TOGETHER

Time (min)	Acetates (%) ^a (1a-c)	Aldehydes (%) ^a (2a-c)
0	100	0
5	52.8 ± 6.5	47.2 ± 6.5
10	41.0 ± 8.5	59.0 ± 8.5
15	22.2 ± 4.4	77.8 ± 4.6

^aMean and standard deviation of three replications.

(a-c) of each fraction (1-3) of the secretion nor the corresponding alcohols (4a-c) produced by the enzyme under nitrogen can be separated from each other by TLC. The alcohols 4a-c were isolated by preparative TLC (eluent 1), and identified by mass spectrometry (Micromass VG 7070). Acetic acid was identified by GLC (25-m OV-1 capillary column, Varian 3700) after derivatization with *N*-methyl-*N*-*t*-butyldimethylsilyl trifluoroacetamide (Schooley et al., 1985) and coinjection with a similarly derivatized authentic sample.

RESULTS

Variation in Proportions of Constituents in Stored Venom. Variations in the proportions of the chemical constituents in the venom depend on how the venom was handled after its collection on paper. Two factors are particularly critical: (1) the nature of the storage solvent, and (2) the time elapsed between emission of the venom and its immersion in solvent. Mixtures of 1a-c, 2a-c, and 3a-c were always found when the storage solvent was *n*-hexane or when the venom was immersed in the solvent 5 or 10 min after its emission. In contrast, only acetates 1a-c were detected by TLC when the venom was placed in CH₂Cl₂ at the very instant of discharge (Figure 1). These observations strongly suggest that *C. scutellaris* uses a defensive mechanism involving the storage of relatively stable acetates and the emission of highly reactive electrophilic aldehydes via enzymatic conversion.

Storage of Precursor in the Dufour Gland. Dufour glands of workers were dissected in H₂O and the glandular fluid briefly extracted with CH₂Cl₂. TLC analyses demonstrated the presence of acetates 1a-c only. The same results were obtained when the dissected glands were crushed on bits of filter paper and kept in contact with air for several hours before extraction. Thus, the acetates are the only precursors stored in the Dufour gland. Their transformation into aldehydes must be mediated by catalysts from another origin.

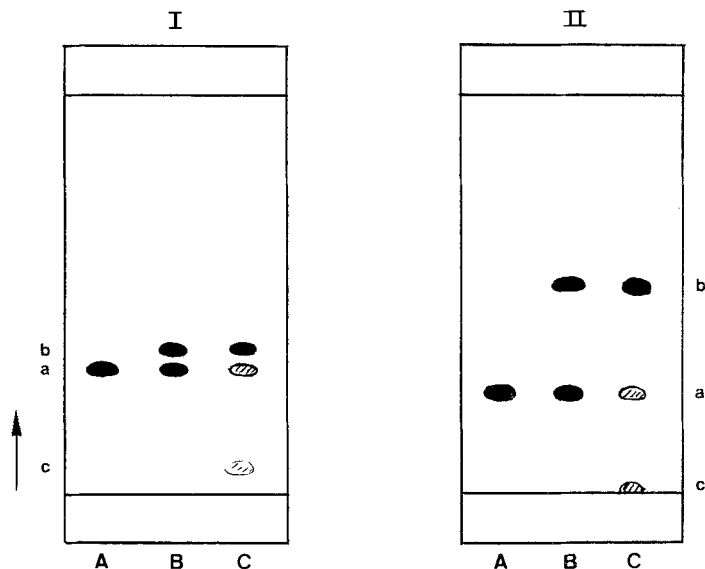


FIG. 1. Redrawn thin-layer chromatographic plates showing the kinetics of ageing of the venom in open air: (A) venom immediately immersed in CH_2Cl_2 ; (B) venom immersed in CH_2Cl_2 after 5 min; (C) venom immersed in CH_2Cl_2 after 15 min; (a) acetates (1a-c); (b) aldehydes (2a-c); (c) acids (3a-c); I, eluent: hexane-acetone, 8:2; II, eluent: CH_2Cl_2 .

Poison Gland as Source of Enzymes Responsible for Aldehyde Production.

In contrast to the above-mentioned results, when dissected Dufour and poison glands were crushed together on a piece of filter paper, left to sit for 15 min, and subsequently extracted with CH_2Cl_2 , TLC analyses showed the presence of mixtures of acetates (1a-c) and aldehydes (2a-c) accompanied by traces of acids (3a-c) (Figure 2). Crushing Dufour glands with pieces of fat body only led to the recovery of the acetates. Thus, the poison gland appears to contain the requisite enzymes to transform the Dufour gland acetates into the corresponding aldehydes. This type of transformation requires two enzymes, an acetate esterase and an alcohol oxidase. The evidence presented below shows that both kinds of enzymes are operative in the defensive secretion of *C. scutellaris*. The fact that only traces of acids were observed suggest that these are not formed by an enzyme-mediated process, but rather by air oxidation of the highly reactive aldehydes. This reactivity is further demonstrated by a spontaneous rearrangement that will be described at the end of this paper.

Identification of Acetic Acid in Secretion. The hydrolysis of acetates 1a-c, triggered by the acetate esterase should produce acetic acid and the alco-

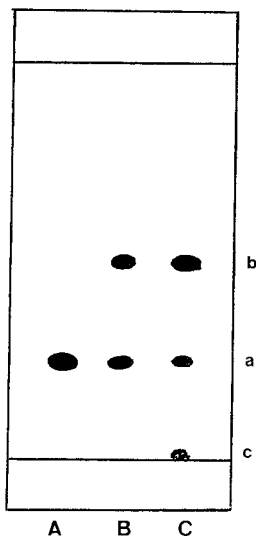


FIG. 2. Redrawn TLC (eluent: CH_2Cl_2) showing the pattern obtained by: (A) extraction of dissected Dufour gland after 15 min; (B) extraction of dissected Dufour and poison gland crushed together for 5 min; (C) as in B for 15 min; (a) acetates (1a-c); (b) aldehydes (2a-c); (c) acids (3a-c).

hols 4a-c. Venom emission by workers is always accompanied by a characteristic odor of acetic acid. The presence of acetic acid in venom samples was indeed demonstrated by GLC of its *t*-butyldimethylsilyl derivative and was always correlated with the presence of the aldehydes (2a-c). The other products of acetate hydrolysis, namely the alcohols 4a-c could never be isolated from crude venom extracts, presumably because their oxidation into the aldehydes is too fast. The alcohols, however, could be trapped under nitrogen.

Accumulation of Alcohols (4a-c) under Nitrogen. The venom, collected on pieces of filter paper, was immediately placed under a nitrogen atmosphere. Under these conditions, TLC analyses of the venom extracts showed that after 1 hr, about 30% of the acetates was transformed into a more polar fraction (Figure 3). The R_f of the latter suggested that it could be the corresponding alcohols (4a-c). Preparative TLC (eluent 1) afforded 0.5 mg of this fraction whose EI-MS (M^{++} at m/z 348 and 346; fragments at m/z 330, 328, 320, 318, 302, 300, . . .) confirmed it contained the alcohols 4a-c.

This result demonstrated that the aldehydes arise from the alcohols under the action of an oxygen-dependent alcohol oxidase. The marked slow-down of acetate hydrolysis under nitrogen (see below and Table 1) may be ascribed to the well-known phenomenon of product inhibition (Mahler and Cordes, 1969),

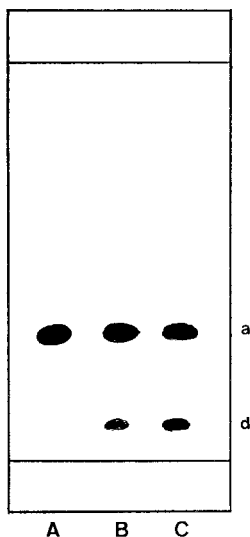


FIG. 3. Redrawn TLC (eluent: hexane-acetone, 8:2) showing the aging of the venom under N_2 : (A) venom immediately immersed in CH_2Cl_2 ; (B) venom after 20 min; (C) venom after 1 hr; (a) acetates (1a-c); (d) alcohols (4a-c).

because the alcohols were not removed from the reaction medium by the oxidase, which is inhibited under N_2 .

Semiquantitative Analysis of Kinetics of Aldehyde Production. The venom, collected on bits of filter paper, was kept in air and dipped in CH_2Cl_2 at different time intervals. TLC analyses of the resulting solutions were performed by photodensitometry at 260 nm. Typical results are shown in Figure 1, and the variations of the percentages of acetates and aldehydes with time are given in Table 1. Furthermore, if the same experiments were run with *n*-hexane as storage solvent, mixtures always resulted, even if attempts were made to stop the transformation at time zero. These results show that the enzyme is still active in hexane. Under our experimental conditions, $47.2\% \pm 6.5$ of the acetates were metabolized into the aldehydes in 5 min (Table 1).

Substrate Specificity of Acetate Esterase. Enzyme activity was first determined *in vitro* on the natural substrates 1a-c in 0.05 M phosphate buffer, in the presence of poison gland extracts. Under these conditions, about 50% of 1a-c were metabolized into the alcohols 4a-c in 20 min. The same experiment performed on *n*-octadecylacetate afforded small amounts of octadecanol, thus showing that the esterase of *C. scutellaris* is not strictly substrate-specific. No aldehydes were produced under these conditions.

Spontaneous Rearrangement of Aldehydes (2a-c). We would like to dis-

cuss here the structure of an artifact that is repeatedly obtained during storage and handling of the secretion of *C. scutellaris*. This artifact, which is less polar than the other derivatives, is obtained in good yields by chromatography of the secretion under daylight on Sephadex LH 20 as described previously (Daloze et al., 1987). It must be derived from the aldehydic fraction (2a-c), since the latter disappears during the chromatographic process.

The spectroscopic properties of the artifact [MS: M^{+} at m/z 346 and 344; UV: λ max 235 nm; IR: ν C=O 1800 cm^{-1} ; ^1H NMR: δ 6.20, 1H, δt , 15.8 and 7 Hz; δ 5.89, 1H, d, 15.8 Hz; δ 5.35, 2H, t, $J = 4.7$ Hz; δ 5.20, 1H, t, $J = 2.6$ Hz; δ 3.27, 2H, m; characteristic signals of a (Z,Z)-diene are also present at δ 6.24, bd, and 5.43, m] strongly suggest its components should have structures 5a-c. This hypothesis was confirmed by decoupling experiments in ^1H NMR ($J_{2,3} = 2.5$ Hz; $J_{5,6} = 15.8$ Hz; $J_{6,7} = 6.5$ Hz; $J_{2,5} = 2.0$ Hz; $J_{3,5} = 1.0$ Hz) and by comparison of the spectral properties of 5a-c with those of model compounds [e.g., α -angelica lactone (Gagnaire and Payo-Subiza, 1963; Hirsch and Szur, 1972)]. The formation of this type of lactone from 4-oxo-2-enals has been well documented (Hirsch and Szur, 1972; Macleod et al., 1977).

Biological Activities of Secretion. Toxicity was measured by counting dead ants (*Myrmica rubra*) observed 48 hr after topical application of 0.1 μl of hexane solution of the compounds. It was not possible to measure the toxicity of pure aldehydes because of their instability. Thus, the toxicity of pure acetates dissolved in hexane was compared to that of the crude secretion kept in dry air during 20 min before its extraction with hexane. The aldehydic content of this secretion was not precisely determined, but substantial amounts of aldehydes together with acetates and acids were detected by TLC (see also Table 1). Only five ants of 20 were found dead 48 hr after 30 μg of the acetates per ant was applied, whereas 19 ants of 20 were dead or paralyzed when the same amount of crude secretion was applied ($P < 0.00001$, Fisher exact probability test). No mortality was observed with pure hexane. It is important to note that 30 μg is about half of what can be collected as a mean in one *Crematogaster* ant by gently squeezing it between fingers (Daloze et al., 1987). Thus, the secretion was more toxic after the enzymatic transformation than before transformation when the acetates alone were present.

C. scutellaris feeding in groups are excited and dispersed in the presence of acetic acid vapors, as demonstrated by placing a bit of filter paper impregnated with acetic acid on a platform 1 cm above ants feeding at its basis.

DISCUSSION

The cooperation between the Dufour and the poison glands in the production of topical poison and alarm pheromone is unique in ants. However, it is worth mentioning that the content of the poison gland of many hymenopterans, includ-

ing ants, is rich in enzymes, among others, esterases (Schmidt, 1982), and the esterase needed to hydrolyze the acetates stored in the Dufour gland is not strongly specific. The uniqueness of the *Crematogaster* system arises more from the particular chemistry of the Dufour gland secretion than from the enzymatic participation of the poison gland. In most ants, the Dufour gland secretion is lipophilic but usually contains much simpler and lighter aliphatic derivatives or sesquiterpenes (Blum and Hermann, 1978; Attygalle and Morgan, 1984).

Similar defensive strategies based on the storage of stable precursors in specialized reservoirs and their transformation by enzymes into highly reactive compounds have already been reported in other arthropods (e.g., Eisner et al., 1963; Schildknecht et al., 1968; Aldrich et al., 1978). The enzymatic transformation of aliphatic acetates into aldehydes by an acetate esterase and an oxygen-dependent alcohol oxidase has been well documented recently in the biosynthesis of aldehydic moth pheromones (Morse and Meighen, 1984, 1986). The enzymes of *Crematogaster* are still active when dissolved in hexane, a well-known property of esterases and related enzymes (Tanaka et al., 1981, Fukui and Tanaka, 1985). However, catalytic activity was totally lost in CH_2Cl_2 , thus indicating that catalysis is due to an enzyme rather than an organic catalyst.

The alarm reaction induced by the vapor of acetic acid is certainly not specific and can be induced by many "nonspecific irritants" (sensu Eisner, 1972), such as volatile acids, aldehydes, or terpenes (Boevé, 1988). However, since acetic acid is produced during poison emission and since it is the only volatile compound detected in the secretion, it is most probably responsible of the alarm pheromonal function of the secretion described by Leuthold and Schlunegger (1973). A more refined analysis of this alarm communication would require a quantification of the emission rate of acetic acid during the enzymatic reaction, which is beyond the scope of this paper.

As expected, the precursor acetates were found to be less toxic than the secretion enriched with the strongly electrophilic aldehydes. The enzymatic transformation of acetates into aldehydes is far from being instantaneous. In our experiments, only half of the acetates was transformed 5 min after their secretion, and significant amounts of acetates still persisted after 15 min. This slow reaction could be in part an artifact of our collecting process, during which we tried to collect as much material as possible by gently squeezing the ants between our fingers. During normal emission, less acetates could be secreted at once and more quickly transformed. However, a "delayed" defense offers obvious advantages, especially since the toxin is unstable. The total conversion of esters to aldehydes in an instant would be an all-or-nothing strategy, because the aldehydes would quickly lose their activity by spontaneous oxidation to acids and rearrangement. On the other hand, a foe contaminated by a mixture that progressively releases its toxins, while penetrating the organism, will be handicapped for a longer time.

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SECONDARY ATTRACTION AND FIELD ACTIVITY OF
BEETLE-PRODUCED VOLATILES IN
Dendroctonus terebrans

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Abstract—Controlled infestation of *Dendroctonus terebrans* (Olivier) on bolts of slash pine, *Pinus elliottii* Engelm. var. *elliottii*, elicited greater attraction of wild conspecifics than uninfested bolts. Secondary attraction was not apparent, however, to standing slash pines that had received volunteer attacks, when compared with attraction to unattacked but susceptible trees. Hindguts from in-flight or attacking female *D. terebrans* contained frontalin, and those from in-flight or attacking males contained *exo*-brevicommin. *Trans*-pinocarveol, *cis*-verbenol, *trans*-verbenol, myrtenal, verbenone, myrtenol, and other compounds were produced by both sexes during gallery construction in host trees. Synthetic frontalin, when deployed with a standard host odor mixture of turpentine and ethanol, was very attractive to male *D. terebrans* in field-trapping experiments. The addition of either *exo*-brevicommin or *endo*-brevicommin to the frontalin-turpentine combination negated the attractive effect of frontalin for males. *Trans*-verbenol, myrtenol, and verbenone had little effect on *D. terebrans* behavior. Responses of females did not differ among treatments in any of the 11 field experiments.

Key Words—*Dendroctonus terebrans*, Coleoptera, Scolytidae, bark beetles, pheromones, primary attraction, secondary attraction, host selection, frontalin, *exo*-brevicommin

INTRODUCTION

In the phases of host selection exhibited by scolytid bark and ambrosia beetles (Wood, 1982), the concentration of colonizing beetles on suitable hosts is purportedly facilitated by pheromones, or "secondary attractants" (after Borden

et al., 1975), from beetles that make the initial selections and those that follow. Pheromones and their behavioral roles are demonstrable and well documented in the relatively few species of tree-killing bark beetles that colonize living trees in large groups over short time periods (reviewed by Borden, 1982). The majority of scolytids, however, colonize recently dead, dying, or severely stressed trees; host-selection behavior has received little research in these species. Some studies of pheromones in species that colonize moribund trees have failed to demonstrate obvious occurrence of beetle-produced attractants or have implicated a greater importance for host odors as attractants (e.g., Lanier, 1983; Millar et al., 1986; Lanne et al., 1987).

The black turpentine beetle, *Dendroctonus terebrans* (Olivier), has been credited with killing healthy pine trees (*Pinus* spp.) that are mechanically injured, as well as those that are stressed or severely weakened (Kowal and Coyne, 1951; Smith and Lee, 1957; Merkel, 1981). Turpentine, the volatile component of pine oleoresin (Mirov, 1961), has been known for some time to be a potent attractant for *D. terebrans* (Hopkins, 1909). Fatzinger (1985) quantified the attraction of *D. terebrans* to turpentine-baited traps over several seasons, and he and Phillips et al. (1988) showed that ethanol would synergistically increase the attraction of beetles to turpentine. Siegfried et al. (1986) reported that no individual monoterpene component of turpentine, nor an artificial mixture of monoterpenes, was as attractive to *D. terebrans* as natural turpentine distilled from oleoresin. The strong attraction exhibited by black turpentine beetles to host odors has led some researchers to suggest that the species does not use pheromones (e.g., Vité et al., 1964; Billings, 1985).

Earlier studies have suggested that black turpentine beetles may use pheromones in host selection or reproductive behavior. Smith (1963) found that *D. terebrans* attacks were more likely to occur on trees previously attacked that season compared to unattacked trees, thus suggesting that secondary attractants may be produced by the initiating attackers. Godbee and Franklin (1976) demonstrated that *D. terebrans* were attracted to trees on which pine bolts were hung that were artificially infested with either male or female *D. terebrans*. Hughes (1973, 1975) identified several terpene oxidation products, some that were known to be pheromones in other scolytids, from hindguts of black turpentine beetles that had been feeding in cut sections of host trees or exposed to vapors of host terpenes. Payne et al. (1987) also identified several known bark beetle pheromones from *D. terebrans* and demonstrated biological activity of some of them in electrophysiological and behavioral studies.

We had three main objectives for the studies reported below. First, we wanted to determine if host material infested with *D. terebrans* is more attractive to wild *D. terebrans* than uninfested host material. Second, we wished to identify volatile compounds produced by *D. terebrans* while attacking host trees.

Third, we investigated the activity of these beetle-produced volatiles as attractants for conspecifics in the field.

METHODS AND MATERIALS

Study Area. All of the field studies described below were conducted on forest land managed by the Owens-Illinois Corporation in Alachua County, Florida, about 14 km east of Gainesville. Slash pine, *Pinus elliottii* Engelm var. *elliottii*, was the predominant commercial tree species and ranged in age from newly planted seedlings to 27-year-old trees in pure, even-aged stands.

Studies of Secondary Attraction. Three separate field studies were conducted to determine if host material infested with *D. terebrans* was more attractive than host material alone. Our first experiment (experiment 1, conducted from July 24 to October 9, 1986) examined the possibility that slash pine trees that were naturally infested with *D. terebrans* were more attractive to wild *D. terebrans* than mechanically injured but uninfested trees. Full-grown slash pines, approximately 30 cm diameter at breast height (DBH; 1.37 m above groundline) and 18 m tall, that had received 8–15 aboveground attacks by *D. terebrans* (as assessed by appropriate-sized entrance holes and resinous “pitch tubes”) were searched for and located near the edge of stands adjacent to logging roads. We were particularly careful not to select trees that had visible evidence of being under attack by other bark- or wood-boring insects, those struck by lightning, or those having experienced similarly obvious physical trauma. For each attacked tree, an unattacked and apparently healthy control tree of similar dimensions and orientation to the stand edge was selected about 20–30 m away. Injuries in the form of axe blazes made at 45° angles into the sapwood from ground line to breast height (BH) were applied to each unattacked tree, the number of axe blazes being equal to the number of visible *D. terebrans* attacks on the corresponding attacked tree. A 16-unit Lindgren funnel trap (Phero Tech Inc., Vancouver, British Columbia, Canada) was placed within 30 cm of each experimental tree by hanging it, facing the logging road, from a PVC pipe standard so that the collection jar was within 10 cm of the ground. Five such attacked–unattacked tree experimental units were established over a six-week period, and beetles caught in traps were collected every two to three days over a four-week period for each unit. Most experimental units were unsuitable after four weeks because either the unattacked trees become attacked by *D. terebrans* or one or both trees in a unit displayed evidence of attacks by other species of bark- or wood-boring insects.

In a second experiment (experiment 2), conducted from June 29 to September 4, 1987, we also studied the response of *D. terebrans* to attacked and

unattacked trees. Each experimental unit was comprised of two mature, apparently healthy, unattacked slash pines that were 20–30 m apart along the edge of a stand bordered by a logging road. Each of the two trees was physiologically stressed by applying about 20 ml of a 4% aqueous solution of the herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) to each of two horizontal axe frills, with lengths equal to one third the bole's circumference, made opposite each other at BH. Such paraquat treatments are known to cause resin-soaking of the xylem tissue around the wound (Roberts, 1973) and make trees attractive to and susceptible to attacks by *D. terebrans* (Siegfried et al., 1986). One of the two trees was then protected to prevent *D. terebrans* attacks by covering the bottom 2 m of the bole with 18 × 16 mesh aluminum screening and by spraying 1 m above the screening, as well as the root collar region below the screening, with a 2% solution of Lindane insecticide (from a 20 EC formulation). Sixteen-unit funnel traps were placed in front of each tree as in the previous experiment. Eight such experimental units (replicates) of protected and unprotected stressed trees were established with no less than 100 m between units. Beetles were collected from traps, and trees were checked for attacks every one to two days. Beetles caught at either tree in a unit were classified as preattack beetles until one or more attacks were observed on unprotected trees, after which trapped beetles were classified as postattack samples. *D. terebrans* response was monitored for four weeks into the postattack period for each unit.

Our last field study of secondary attraction (experiment 3), conducted from June 12 to June 24, 1987, was an experiment in which the attractiveness of slash pine log bolts that were artificially infested with *D. terebrans* was compared with that of uninfested bolts. Two mature trees of similar dimensions were felled, and two bolts, each 130 cm long by 25–30 cm diam., were cut from the basal section of each tree; cut ends were immediately placed in buckets of water to prevent excessive drying, and the bolts were returned to the laboratory. Wild *D. terebrans* were live-captured in funnel-traps baited with a mixture of turpentine and ethanol (see below). Thirteen 4-mm holes for introducing beetles were drilled to the sapwood at 45° angles on each of the four bolts and the following beetle-infestation treatments were randomly assigned: (1) 13 females, (2) 13 males, (3) 13 females with 13 males introduced 12 hr later, and (4) 13 holes with no beetles as the control. Beetles were confined in holes with 4 × 4-cm pieces of screening and all four bolts were securely covered with 18 × 16 mesh aluminum screening to prevent attacks by other insects. Bolts were taken to the field and deployed in traps for the experiment. We used a modification of Fatzinger's (1985) bounce column trap, in which the blackened stovepipe was replaced with our screened bolt. Each bolt was placed upright in a plastic wading pool (1.2 m diam. by 23 cm deep) filled with 20 liters of soapy water and stabilized with three guy wires from the top of the bolt to side of the pool. The trap was designed so that *D. terebrans* and other responding insects

would hit the screened bolt and then drop into the soapy water. Experimental bolts were randomly assigned to four traps spaced 40 m apart in a line along the edge of a 20-year-old stand. Bolts were kept in place for two days, after which trapped beetles were collected and the bolts randomly reassigned to the traps. Five such two-day trapping periods were conducted.

Extraction and Identification of Beetle Volatiles. Wild *D. terebrans* were either live-trapped in flight or excised from galleries in naturally attacked trees during the summers of 1986 and 1987 for gas chromatographic (GC) analysis of volatiles present in their hindguts. Upon collection, beetles were immediately placed in a cooler at 5°C and returned to the laboratory for dissection and extraction. Volatiles from individual beetles were extracted by removing the hindgut and associated tissues (part of the midgut, malpighian tubules, and some fat body), placing it in 20 μ l of chilled pentane in a 1.5-ml screw-top septum vial (Pierce Chem. Co., Rockford, Illinois), macerating briefly with a probe, and sonicating for 15 sec in a sonicating cleaner. An internal standard of 3-octanol (424 ng) was added at the time of extraction for GC quantification of volatiles in individual beetle samples. Groups of 5–10 hindguts were pooled in other samples and extracted in the same way with about 10 μ l of pentane per hindgut.

Volatiles extracted from hindguts were analyzed on a Varian 3700 GC equipped with a flame ionization detector and a Hewlett Packard 3390A recording integrator. Two different capillary GC columns were used in the splitless mode under the following conditions: (1) a 10-m \times 0.53-mm Superox FA fused silica column (Alltech Associates, Inc., Deerfield, Illinois), injector 160°C, detector 180°C, column held at 60°C for 10 min then programmed at 1°/min to 100°C and held for 20 min, He carrier gas flow at 34.0 cm/sec at 100°C; (2) a 30-m \times 0.25-mm DB-1 fused silica column (J and W Scientific, Inc., Folsom, California), injector 180°C, detector 250°C, column held at 50°C for 12 min then programmed at 2°/min to 75°C and held for 45 min, the flow at 25.1 cm/sec at 100°C. Identifications were initially made on GC by comparing retention times of unknowns on each column with those of available standards that we suspected were present in *D. terebrans*. Our GC identifications were confirmed and additional compounds identified in a few representative samples using coupled GC–mass spectroscopy (MS). Two different GC–MS instruments were used, each with a 30-m DB-1 column.

Field Activity of Synthetic Beetle Volatiles. Eight separate trapping experiments were conducted in 1986 and 1987 to assess the activity of synthetic formulations of volatiles identified from *D. terebrans*. Source, purity, release devices, and release rates of test compounds are given in Table 1; details of the individual experiments are given in Table 2. In addition to field-testing compounds found in *D. terebrans* hindguts, we also tested *endo*-brevicomins, which was reported from male *D. terebrans* by Payne et al. (1987). In experiments

TABLE 1. SOURCE, PURITY, RELEASE DEVICES AND RELEASE RATES OF VOLATILE MATERIALS USED IN TRAPPING STUDIES OF *D. terebrans*

Material	Source	Purity (%) ^a	Release device	Release rate (mg/24 hr) ^b
Turpentine ^c	Shelton Naval Stores Processing Co., Valdosta, GA	99: monoterpene hydrocarbons ^d	250-ml Nalgene bottle with 5 cm of 15-cm cotton dental wick extending through cap	10, 140.00 ^e
(±)-Frontalin	Phero Tech Inc., Vancouver, BC	>99	2-ml screw-cap glass vial with 2-mm hole in cap ^e	5.77
(±)- <i>exo</i> -Brevicommin	Phero Tech Inc.	98	2-ml screw-cap vial	5.05
(±)- <i>endo</i> -Brevicommin	Phero Tech Inc.	>99	2-ml screw-cap vial with 2-mm hole in cap ^e	3.93
(±)- <i>trans</i> -Verbenol	Phero Tech Inc.	80	Two open 2-ml screw-cap vials ^e	4.40
(-)-Myrtenol	Aldrich Chemical Co., St. Louis, MO	98	Two open 2-ml screw-cap vials ^e	1.82
(-)-Verbenone	SCM Organics, Jacksonville, FL	93	One open 2-ml screw-cap vial ^e	4.17

^a Determined by GC on 30 m DB-1 column (see Methods and Materials).

^b Approximate rates determined in the laboratory at 27°C by quantitative GC analysis of bait volatiles trapped on Tenax GC; values represent means for five devices. Release rate of turpentine from Phillips et al. (1988).

^c Turpentine was mixed 1:1 (v/v) with 95% ethanol.

^d Majority of turpentine was a mixture of monoterpene hydrocarbons, of which α -pinene (66.5%) and β -pinene (29.4%) were the major components (see Phillips et al., 1988, for details).

^e All glass vial devices were loaded with 20 μ l of material, neat.

TABLE 2. DETAILS OF FIELD TRAPPING EXPERIMENTS IN WHICH RESPONSES OF *D. terebrans* TO SYNTHETIC VOLATILES WERE STUDIED

Experiment	Objectives ^a	Treatments and setup ^a	Results reported
4	Determine activity of F and nB	T, F, nB, T+F, T+nB using funnel traps; five 1-day replicates, June 16-21, 1986	Table 6
5	Determine effect of combining F with nB	T, T+F, T+nB, T+F+nB using funnel traps; four 3-day replicates, June 27 to July 7, 1986	Table 7
6	Determine activity of xB	T, xB, T+xB using funnel traps; five 2-day replicates, June 27 to July 7, 1986	Table 8
7	Determine effect of F, xB, and nB in combinations	T, T+F, T+F+xB, T+F+nB, T+F+xB+nB using funnel traps; eight 1-day replicates, June 16-26, 1987	Table 9
8	Determine activity of tV, M, and V	T, tV, M, V, T+tV, T+M, T+V using funnel traps; seven 1-day replicates, July 9-17, 1986	Table 10
9	Determine effect of V on activity of F and T	T, T+V, T+F, T+F+V using funnel traps; four 2-day replicates, August 11-20, 1986	Table 11
10	Determine effect of tV and M on activity of F	T+F, T+F+tV, T+F+M using bounce column traps; four 2-day replicates September 24 to October 3, 1986	Table 12
11	Determine effect of tV and M in combination on activity of F	T, T+F, T+F+tV, T+F+M, T+F+tV+M using funnel traps; five 2-day replicates, October 14-26, 1987	Table 13

^aTreatment abbreviations: T = mix of turpentine and ethanol, F = frontalin, nB = *endo*-brevicommin, xB = *exo*-brevicommin, tV = *trans*-verbenol, M = myrtenol, V = verbenone.

with synthetics, we utilized a 1:1 mixture (v/v) of turpentine and 95% ethanol as a representative host odor attractant (as in Phillips et al., 1988). We also used 16-unit funnel traps hung from PVC pipe standards for these experiments, except for experiment 10, in which we used bounce column traps (Fatzinger,

1985) to maximize beetle catches. Traps were positioned 20–30 m apart in recent clear-cut areas adjacent to residual mature (20–25 years) stands of slash pines. Each experiment used as many traps as there were treatments. Treatments (baits) were randomly assigned to traps for a given time period (one to three days), after which trapped beetles were collected; baits were replaced with fresh material and treatments randomly assigned again to traps so that each trapping period represented a block in a randomized block design. Synthetic beetle volatiles were evaporated separately from small glass vials (Table 1) that were secured in wooden holders equipped with a small aluminum roof to protect them from excessive sun and rain. Vial holders and turpentine-ethanol dispensers were hung from the fourth funnel from the bottom (about 45 cm above ground line) on funnel traps, or from the top of the stovepipe (about 1.2 m above ground line) on bounce column traps.

Data Analysis. Data on field responses of *D. terebrans* were processed using the Statistical Analysis System package. Because of the occurrence of many zero counts, raw data from each experiment were transformed by the calculation of $y = \sqrt{x + 0.5}$. Transformed data were then subjected to analysis of variance followed by means comparisons with the Student-Newman-Keuls test (for experiments with three or more treatments) or Students' *t* test (for experiment with two treatments). Differences in the sex ratio of beetles responding to different treatments were analyzed by computing the percentage of females responding per replicate for each treatment, transforming percentages via $y = \arcsin\sqrt{\%x}$, and then performing analysis of variance and means comparisons as before.

RESULTS

Naturally attacked and mechanically injured slash pines were similarly attractive to *D. terebrans* (Table 3). Although the total number of beetles responding to the five attacked trees was about twice that responding to injured trees over the four-week period (228 vs. 115), differences in numbers caught were not statistically significant (e.g., $P = 0.24$ for sexes combined). In our second experiment (Table 4), protected and unprotected stressed trees were equally attractive prior to *D. terebrans* attacks (an average period of $14.3 \pm 3.7 \text{ SE}_{\bar{x}}$ days), and lack of a difference in attractiveness persisted after the unprotected trees each received two to three *D. terebrans* attacks. Thus, these first two experiments provided no evidence for secondary attraction due to attacking beetles. Results from known numbers of *D. terebrans* artificially introduced into cut bolts (Table 5), however, did demonstrate secondary attraction of *D. terebrans* to conspecifics on host material. Although responses of

TABLE 3. RESPONSE OF *D. terebrans* OVER FOUR-WEEK PERIOD TO SLASH PINES THAT WERE NATURALLY ATTACKED OR MECHANICALLY INJURED BUT NOT ATTACKED IN EXPERIMENT 1

Tree type	Mean ($SE_{\bar{x}}$) number caught at trees ^a		Mean % females ^a
	Females	Males	
Attacked	23.8 (11.8)	21.8 (9.4)	49.1 (3.3)
Injured controls	9.2 (6.7)	11.8 (8.6)	35.2 (10.3)

^aThe *t* tests detected no significant differences ($P > 0.05$) in the number of either sex or the percentage of females trapped at either type of tree ($N = 5$).

TABLE 4. RESPONSE OF *D. terebrans* TO PARAQUAT-TREATED SLASH PINES PROTECTED OR UNPROTECTED FROM ATTACKS IN EXPERIMENT 2

Tree type	Preattack period: Mean ($SE_{\bar{x}}$) number caught per day at trees ^a		Mean % female
	Females	Males	
Protected	7.3 (5.7)	6.4 (4.7)	54.7 (7.0)
Unprotected	3.6 (1.6)	5.2 (2.6)	50.2 (7.7)
Tree type	Postattack period: Mean ($SE_{\bar{x}}$) number caught in first week ^a		Mean % female
	Females	Males	
Protected	5.0 (1.6)	5.5 (1.6)	40.8 (6.8)
Unprotected (attacked)	7.4 (4.7)	7.3 (3.9)	36.0 (8.7)

^aThe *t* tests detected no significant differences ($P > 0.05$) in the number of either sex or the percentage of females trapped at each type of tree in either the preattack or postattack periods ($N = 8$).

TABLE 5. RESPONSE OF *D. terebrans* TO SLASH PINE BOLTS ARTIFICIALLY INFESTED WITH CONSPECIFIC BEETLES IN EXPERIMENT 3

Treatment	Mean ($SE_{\bar{x}}$) number caught per replicate ^a		Mean % females ^a
	Females	Males	
Bolt only	2.0a (0.7)	0.8a (0.4)	56.0ab (17.7)
Bolt + 13 males	4.6a (2.4)	3.4ab (2.1)	73.4a (11.5)
Bolt + 13 females	1.4a (0.9)	5.2bc (1.9)	12.6b (5.7)
Bolt + 13 females + 13 males	3.6a (1.7)	7.8c (2.4)	21.2b (8.8)

^a Means in a column with the same letter are not significantly different ($P > 0.05$, Student-Newman-Keuls test, $N = 5$).

females were not significantly different among treatments, males displayed higher responses to bolts with females only or females plus males, over blank bolts or bolts with males only. The sex ratios of responding beetles were strongly in favor of males for treatments containing females and in favor of females for bolts containing just males.

GC analyses of hindguts from individual beetles trapped in flight (Figure 1) revealed that females contained one major volatile compound, the bicyclic ketal frontalinalin, and males contained another major volatile, the bicyclic ketal *exo*-brevicominalin. Beetles that were actively constructing galleries in living trees contained a complex mixture of volatiles in pentane extracts of hindguts (Figure 2). Again, females produced frontalinalin and males contained *exo*-brevicominalin, but hindgut extracts from both sexes contained similar complements of monoterpene hydrocarbons, presumably from the host trees' oleoresin, and the following oxygenated monoterpenes: *trans*-pinocarveol, *cis*-verbenol, *trans*-verbenol, myrtenal, verbenone, and myrtenol. Identifications of the bicyclic ketals and the oxygenated terpenes were confirmed with GC-MS analyses. These compounds were looked for but never found in extracts of slash pine oleoresin. Not all of the compounds reported here were found in every *D. terebrans* sample analyzed (e.g., *cis*-verbenol was not detected in the female extract shown in Figure 2), but every beetle collected from active galleries had detectable levels of *trans*-verbenol and myrtenol. On occasion, we detected up to seven minor components in both sexes, mostly eluting in the region of the terpene alcohols, but these have yet to be identified. Details of quantitative estimates of volatiles

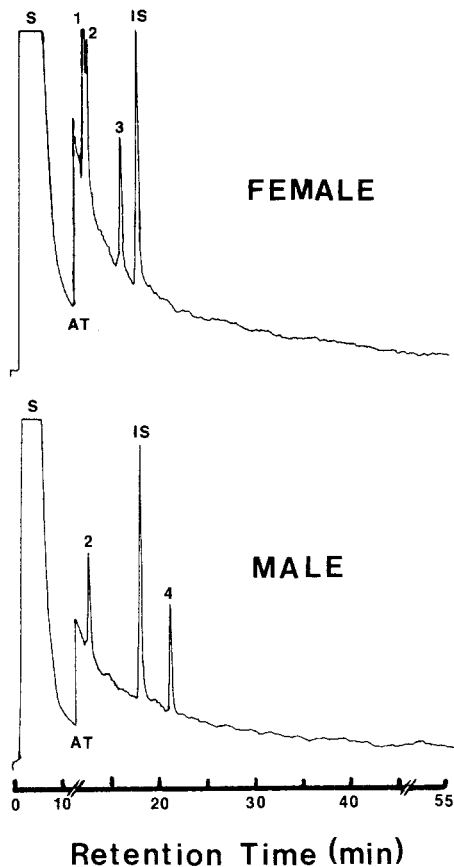


FIG. 1. GC records (DB-1 column) of single-hindgut pentane extracts from one female (top) and one male (bottom) *D. terebrans* captured in flight June 2, 1987; S = solvent peak, AT = sensitivity of recording integrator increased by two orders of magnitude, IS = internal standard of 3-octanol. Identities of peaks and estimated sample quantities of beetle compounds are: 1, frontalin, 462.9 ng; 2, α -pinene; 3, β -pinene; 4, *exo*-brevicommin, 170.8 ng.

produced by individual *D. terebrans* during gallery construction will be reported elsewhere.

Frontalin was a potent attractant for male *D. terebrans* when deployed with a turpentine-ethanol mixture (Tables 6, 7, and 9). In experiment 1 (Table 6), females displayed no significant difference in response among the five treatments, but males were significantly attracted to turpentine plus frontalin, and their response to turpentine plus *endo*-brevicommin was not different from that to

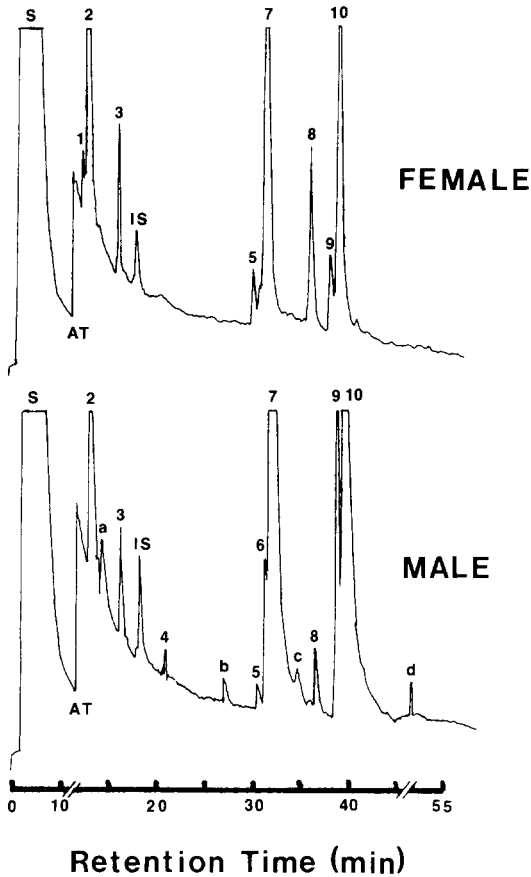


FIG. 2. GC records (DB-1 column) of single-hindgut pentane extracts from one female (top) and one male (bottom) *D. terebrans* excised from a 7-day-old gallery in a slash pine, June 2, 1987; S = solvent peak, AT = attenuator of recording integrator increased by two orders of magnitude, IS = internal standard of 3-octanol. Identities of host-derived peaks in both records are 2, α -pinene; 3, β -pinene; peaks a, b, c, and d in the male record are unknowns. Identities and estimated sample quantities of peaks in the female record are: 1, frontalinal, 127.7 ng; 5, *trans*-pinocarveol, 476.9 ng; 7, *trans*-verbenol, 11,708.9 ng; 8, myrtenal, 1929.5 ng; 9, verbenone, 925.8 ng; 10, myrtenol, 5071.0 ng. Peak identities and estimated quantities in the male record are: 4, *exo*-brevicommin, 66.1 ng; 5, *trans*-pinocarveol, 74.9 ng; 6, *cis*-verbenol, 377.6 ng; 7, *trans*-verbenol, 15,532.0 ng; 8, myrtenal, 227.5 ng; 9, verbenone, 1578.4 ng; 10, myrtenol, 8738.2 ng.

TABLE 6. RESPONSE OF *D. terebrans* TO TURPENTINE (T), FRONTALIN (F), AND *endo*-BREVICOMIN (nB) IN EXPERIMENT 4

Treatment	Mean (SE \bar{x}) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T	5.4a (2.5)	1.6ab (0.5)	66.5a (17.2)
F	0a	0.4a (0.2)	0b
nB	0.2a (0.2)	0a	20.0ab (20.0)
T + F	3.4a (1.1)	13.0c (4.0)	20.7ab (6.8)
T + nB	7.0a (2.7)	3.4b (1.1)	53.1ab (14.5)

^aMeans in a column with the same letter are not significantly different ($P > 0.05$, Student-Newman-Keuls test, $N = 5$).

turpentine only. Frontalin and turpentine each displayed low attractiveness when deployed singly, but the increased attractiveness of their combination indicates a synergistic effect for males. In experiment 5 (Table 7), females again displayed similar low responses to all treatments, while males were attracted to

TABLE 7. RESPONSE OF *D. terebrans* TO TURPENTINE (T), FRONTALIN (F), AND *endo*-BREVICOMIN (nB) IN EXPERIMENT 5

Treatment	Mean (SE \bar{x}) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T	2.8a (1.4)	4.3a (1.8)	38.9a (4.9)
T + nB	2.5a (1.5)	1.3a (0.3)	41.4a (23.9)
T + F	3.3a (1.7)	29.5b (13.4)	12.3a (3.2)
T + F + nB	5.0a (1.7)	7.3a (2.6)	30.8a (10.7)

^aMeans in a column with the same letter are not significantly different ($P > 0.05$, Student Newman-Keuls test, $N = 4$).

the combinations of turpentine and frontalin. Addition of *endo*-brevicommin to the frontalin-turpentine mixture reduced the response of males to the level of that to turpentine only. Although *exo*-brevicommin exhibited no obvious attractive effect for males or females when deployed either singly or in combination with turpentine (experiment 6, Table 8), it did significantly reduce the attractiveness of turpentine plus frontalin in a manner similar to that of *endo*-brevicommin (experiment 7, Table 9). Here again, females did not display significantly different responses among the treatments deployed. The combination of the two brevicommin isomers had the same effect on turpentine-frontalin activity as did either applied individually.

Trans-verbenol, myrtenol, and verbenone showed little or no attractive activity for *D. terebrans* (Table 10). The combinations of *trans*-verbenol or myrtenol each with turpentine did not significantly alter beetle response over that to turpentine only. The response of males to a combination of verbenone and turpentine was lower than the response of males to just turpentine; this result was not corroborated in experiment 9 (Table 11). Verbenone decreased the response of males to frontalin and turpentine, but this effect was not statistically significant. Neither *trans*-verbenol nor myrtenol significantly affected response of beetles to turpentine plus frontalin (experiment 10, Table 12). The combination of *trans*-verbenol and myrtenol with frontalin and turpentine was more attractive to male *D. terebrans* than *trans*-verbenol with turpentine and frontalin (experiment 11, Table 13). As before, the responses of female *D. terebrans* did not differ among treatments.

TABLE 8. RESPONSE OF *D. terebrans* TO TURPENTINE (T) AND *exo*-BREVICOMMIN (xB) IN EXPERIMENT 6

Treatment	Mean (SE \bar{x}) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T	1.6ab (0.8)	1.0ab (0.6)	41.3a (19.4)
xB	0.4a (0.2)	0a	40.0a (24.5)
T + xB	2.6b (0.8)	1.8b (0.8)	58.9a (7.2)

^aMeans in a column with the same letter are not significantly different ($P > 0.05$, Student-Newman-Keuls test, $N = 5$).

TABLE 9. EFFECTS OF *exo*-BREVICOMIN (xB) AND *endo*-BREVICOMIN (nB) ON RESPONSE OF *D. terebrans* TO TURPENTINE (T) AND FRONTALIN (F) IN EXPERIMENT 7

Treatment	Mean (SE \bar{x}) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T	3.0a (1.0)	3.0a (0.5)	43.3a (8.1)
T + F	1.5a (0.6)	16.5b (4.7)	5.5b (1.8)
T + F + xB	3.3a (2.0)	5.3a (1.9)	19.1bc (7.7)
T + F + nB	3.8a (1.3)	7.0a (1.6)	32.1ac (8.1)
T + F + xB + nB	5.0a (2.3)	5.5a (2.1)	36.7ac (8.4)

^aMeans in a column with the same letter are not significantly different ($P > 0.05$, Student-Newman-Keuls test, $N = 8$).

TABLE 10. RESPONSE OF *D. terebrans* TO TURPENTINE (T), *trans*-VERBENOL (tV), MYRTENOL (M), AND VERBENONE (V) IN EXPERIMENT 8

Treatment	Mean (SE \bar{x}) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T	1.3a (0.6)	1.3a (1.1)	51.8a (16.3)
tV	0b	0b	0a
M	0b	0b	0a
V	0.3b (0.2)	0b	28.6a (18.4)
T + tV	1.4a (1.1)	1.0ac (0.6)	45.3a (17.3)
T + M	1.9a (1.0)	1.6a (0.9)	43.4a (14.5)
T + V	0.9a (0.9)	0.3bc (0.3)	10.7a (10.7)

^aMeans in a column with the same letter are not significantly different ($P > 0.05$, Student-Newman-Keul test, $N = 7$).

TABLE 11. EFFECTS OF VERBENONE (V) ON RESPONSE OF *D. terebrans* TO TURPENTINE (T) AND FRONTALIN (F) IN EXPERIMENT 9

Treatment	Mean ($SE_{\bar{x}}$) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T	1.3a (0.5)	0.5a (0.3)	54.2a (20.8)
T + V	1.0a (0.4)	1.5ab (0.5)	35.0a (11.9)
T + F	1.3a (0.7)	4.0b (0.9)	25.0a (10.8)
T + F + V	0.5a (0.5)	1.5ab (0.9)	8.3a (8.3)

^aMeans in a column with the same letter are not significantly different ($P > 0.05$, Student-Newman-Keuls test, $N = 4$).

TABLE 12. EFFECTS OF *trans*-VERBENOL (tV) AND MYRTENOL (M) ON RESPONSE OF *D. terebrans* TO TURPENTINE (T) AND FRONTALIN (F) IN EXPERIMENT 10

Treatment	Mean ($SE_{\bar{x}}$) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T + F	1.0 (0.7)	9.0 (4.8)	6.4 (3.8)
T + F + tV	1.3 (0.3)	5.0 (1.5)	22.9 (5.1)
T + F + M	2.5 (0.9)	9.0 (1.0)	20.2 (7.3)

^aNo significant differences detected among means in a column ($P > 0.05$, Student-Newman-Keuls test, $N = 4$).

DISCUSSION

Our experiment with artificially infested bolts provided good evidence that *D. terebrans* on a host can induce a secondary attraction of conspecifics. Secondary attraction was not clearly manifested in studies with standing trees, but several caveats diminish the importance of these results. With naturally attacked trees, we could not control the "quality" of the trees by knowing the exact numbers of attacks, ages of attacks, numbers of each sex attacking, and type

TABLE 13. EFFECTS OF *trans*-VERBENOL (tV) AND MYRTENOL (M) ON RESPONSE OF *D. terebrans* TO TURPENTINE (T) AND FRONTALIN (F) IN EXPERIMENT 11

Treatment	Mean (SE \bar{x}) number caught per replicate ^a		Mean % females ^a
	Females	Males	
T	4.6a (1.3)	8.6ab (2.5)	36.4a (4.6)
T + F	4.6a (1.1)	14.0ab (3.6)	28.6a (6.9)
T + F + tV	2.2a (1.7)	6.6a (3.5)	16.9a (7.3)
T + F + M	4.4a (1.9)	14.6ab (6.4)	21.4a (6.8)
T + F + tV + M	5.4a (1.5)	20.6b (4.4)	19.8a (3.3)

^aMeans in a column with the same letter are not significantly different ($P > 0.05$, Student-Newman-Keuls test, $N = 5$).

or intensity of agent(s) that predisposed the trees to attack. In paraquat-treated trees, we partially controlled the predisposing stress on the tree but not the timing or number of attacks. Moreover, we feel that the level of host odors from these stressed and injured trees may have been so high and attractive as to override any effects of beetle attacks on them. The lower levels of host odors from artificially infested bolts probably allowed the effects of attacking beetles to be detected.

The volatiles we identified from *D. terebrans* hindguts are typical of compounds found in other *Dendroctonus* species. We never detected the compound *endo*-brevicommin, although it was reported from *D. terebrans* males by Payne et al. (1987). *D. terebrans* in north Florida may not produce *endo*-brevicommin, but we cannot rule out transient production that went unsampled or production at levels below our detection sensitivity. The terpenoids *trans*-pinocarveol, *cis*-verbenol, *trans*-verbenol, myrtenal, verbenone, and myrtenol that we found in both sexes are common metabolic products of pine bark beetles feeding on host material or simply exposed to vapors of host monoterpenes (e.g., Hughes, 1973, 1975). If these terpenoids prove to have little or no behavioral activity for *D. terebrans*, as our field tests with three of them suggest, they may simply represent metabolic detoxification products of the beetles (White et al., 1980) and/or associated microorganisms (Brand et al., 1975; Conn et al., 1984).

We repeatedly showed that frontalin, a female-produced compound, was very attractive to male *D. terebrans* when it was deployed with a turpentine-

ethanol mix. The male-produced *exo*-brevicommin was not behaviorally active alone or with turpentine but had the effect of blocking the activity of frontalin and turpentine for males. This effect was also seen for *endo*-brevicommin, although this compound was not detected in our experimental beetles. The attractive nature of frontalin with turpentine and the blocking effect of *endo*-brevicommin were demonstrated earlier by Payne et al. (1987). The apparently equal activity of *exo*- and *endo*-brevicommin suggests that *D. terebrans* do not distinguish between the two geometric isomers. The bicyclic ketals used for these experiments were racemic formulations. Pure enantiomers, or the naturally occurring ratio of enantiomers, may elicit different responses from *D. terebrans*, as was shown for *D. frontalis* (Vité et al., 1985). Additionally, different release rates of these compounds may elicit different behavioral responses.

The synthetic oxygenated terpenes had little behavioral effect on responses of *D. terebrans* compared with those of the bicyclic ketals. Verbenone may have an inhibitory or blocking effect against male response to attractants, but this will require more rigorous testing. Verbenone is a reputed inhibitor or "antiaggregation pheromone" in a number of scolytids (Borden, 1982). We found no evidence that *trans*-verbenol enhances the response of *D. terebrans* to turpentine, as Fatzinger et al. (1987) reported. In fact, our data show decreases in the responses of *D. terebrans* to traps containing *trans*-verbenol compared with those to other treatments, but these differences were not always significant. The supplier of our synthetic *trans*-verbenol indicated that it contained less than 0.3% verbenone. If verbenone is an inhibitor of response by *D. terebrans* males, it is possible that small amounts of verbenone present as a contaminant in the *trans*-verbenol, or verbenone produced via autooxidation (Borden et al., 1986) of *trans*-verbenol in baits, could account for lowered responses to *trans*-verbenol. As with other semiochemicals, release rate and enantiomeric composition of *trans*-verbenol could be important variables affecting beetle behavior.

Clearly, *D. terebrans* utilizes pheromones in its behavioral repertoire; yet our studies and those of others point to the very strong attraction of these beetles to host odors. We trapped relatively high numbers of *D. terebrans* at physiologically stressed and injured trees (Table 4), whether or not they were attacked. *D. terebrans* does not exhibit a "mass attack" of the magnitude seen in more aggressive scolytids for which pheromones play an important role in host selection. Smith (1963) reported an average of 28.6 attacks per tree by *D. terebrans* over an entire season; compare this with attack densities of 73 attacks/m² for *D. ponderosae* (Raffa and Berryman, 1983) or 5–10 attacks/100 cm² for *D. frontalis* (Fargo et al., 1978) over several meters of bole surface within days or weeks. The importance of pheromones, therefore, in host location by *D. terebrans* is not straightforward.

Pheromones of *D. terebrans* may be more important in sexual behavior

than in host-selection behavior. The idea that many bark beetle pheromones could be considered sex pheromones was suggested earlier by Alcock (1982). In each of our eight experiments with synthetic volatiles and one with infested bolts, only males displayed different behavioral responses to the various treatments; females responded to all treatments equally. Research to date indicates that both sexes of *D. terebrans* will fly to suitable host trees in response to host odors. Our studies lead us to suggest that males are attracted to females by the frontalin they produce when on a host, and that response of other males to frontalin can be interrupted by *exo*-brevicomin released by males that arrived earlier. Since we showed significant responses only by males to frontalin and *exo*-brevicomin, we feel it is appropriate to refer to these compounds as sex pheromones, rather than as aggregation or antiaggregation pheromones.

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BEHAVIORAL ACTIVITY OF OPTICAL ISOMERS OF
5,9-DIMETHYLHEPTADECANE, THE SEX
PHEROMONE OF *Leucoptera scitella* L.
(LEPIDOPTERA: LYONETIDAE).

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Abstract—Among the pure stereoisomers of 5,9-dimethylheptadecane, a previously identified sex pheromone component of *Leucoptera scitella* L., only the *S,S* isomer yielded trap captures in the field. The addition of the other stereoisomers had no effect on catches. The addition of low percentages of racemic 5,9-dimethylhexadecane, a previously identified minor component in the sex pheromone, did not influence trap catches or alter behavior of males approaching an attractant source in the field.

Key Words—Mountain-ash bentwing, *Leucoptera scitella*, Lepidoptera, Lyonetidae, (*R,R*)-5,9-dimethylheptadecane, (*R,S*)-5,9-dimethylheptadecane, (*S,R*)-5,9-dimethylheptadecane, (*S,S*)-5,9-dimethylheptadecane, 5,9-dimethylhexadecane, trapping, behavior, sex pheromone, asymmetric synthesis.

INTRODUCTION

The sex pheromone of the mountain-ash bentwing, *Leucoptera scitella* L. (Lepidoptera: Lyonetiidae) has recently been identified as 5,9-dimethylheptadecane (Francke et al., 1987). Although a synthetic mixture of all four possible ster-

eoisomers captured significant numbers of male *L. scitella* in field trapping tests, it was not determined which of the optical isomer(s) carries biological activity.

Also, during the previously mentioned identification project, small amounts of 5,9-dimethylhexadecane were found to be present in pheromone collections (Francke et al., 1989). No attempt has been made so far to determine the biological activity of this compound.

The objective of the present study was to study whether only one or more of the pure stereoisomers were needed for behavioral activity and whether the addition of the racemic minor component influenced activity of the active isomer(s) of the main component in the field.

METHODS AND MATERIALS

Chemicals. The four stereoisomers of 5,9-dimethylheptadecane were synthesized (Leikauf, 1988) by coupling enantiomerically >99.8% pure (*R*)- and (*S*)-2-methyldecyl bromide with (*R*)- and (*S*)-3-methylheptanal. Preparations of these compounds involved as key steps asymmetric alkylations of chiral propionates (Schmierer et al., 1981) and conjugate additions of butyl copper complexes to chiral crotonates (Helmchen and Wegner, 1985). Products of these reactions were purified by medium-pressure liquid chromatography to ensure diastereomeric purities of >99.9%. The coupling was carried out by a Grignard reaction. The resultant hydroxyl groups were removed by reduction of the corresponding mesylates with lithium triethylborohydride. Stereoisomeric purities of the final products must be >99.8%, as only synthetic procedures not involving bonds directly attached to the chirality centers were employed.

Chemical purity was determined by GLC-MS (Hewlett-Packard 5890 A/5970; fused silica capillary column DB-1, 30 m × 0.3 mm). Each of the stereoisomeric 5,9-dimethylheptadecanes contained traces of hydrocarbons introduced in the reduction step by the lithium triethylborohydride solution employed (Aldrich). Optical rotations were measured on a Perkin-Elmer polarimeter 241 (temperature: 21°C).

(*R,R*)-5,9-Dimethylheptadecane was 98% pure, $[\alpha]_D = -2.1$ ($c = 2.1$, chloroform).

(*5R,9S*)-5,9-Dimethylheptadecane is denoted *R,S* isomer in the following purity: 97%, $[\alpha]_D = -1.5$ ($c = 2$, chloroform).

(*5S,9R*)-5,9-Dimethylheptadecane is denoted *S,R* isomer in the following purity: 96%, $[\alpha]_D = 1.1$ ($c = 2.1$, chloroform).

(*S,S*)-5,9-Dimethylheptadecane is denoted *S,S* isomer in the following purity: 96%, $[\alpha]_D = 2.1$ ($c = 2$, chloroform).

Pure racemic mixtures of 5,9-dimethylheptadecanes and -hexadecanes were obtained from Prof. W. Francke (Hamburg, F.R.G.).

Trapping Tests. Test traps of similar design to Tetra traps with flaps (Arn

et al., 1979), but made from transparent polyethylene sheets, were used. Chemicals were applied in 10- μ l hexane solutions to 1-cm pieces of rubber tubing (commercial product, obtained from Borászati Szaküzlet, Budapest). Traps in one replicate were hung at about 1.5 m in the foliage of apple trees, at ca. 2–3 m from each other, composing a rectangle. The positions of the traps within the rectangle were changed clockwise every one to two days (Érd), or once weekly (Dunaharaszti, Halásztelek). On these occasions trapped moths were counted and sticky inserts renewed. The distance between replicates ranged from 30 to 100 m.

At Érd (Pest County, Hungary) and Halásztelek (Pest County, Hungary) tests were set up in commercial apple orchards, while at Dunaharaszti they were placed in backyard gardens having various orchard trees and vegetables apart from apple trees.

Direct Observation of Male Behavior. Studies of male behavior close to the attractant source in the field were conducted using a 20 \times 20-cm flat, transparent plastic sheet. The dispenser with the attractant was attached to a slot in the middle of this platform. The sheet was placed into the crown of a tree at the height of ca. 1.5 m, and the behavior of approaching males was described into a cassette recorder, then transcribed in the lab. The observer was stationed at 1–2 m crosswind from the sheet.

Observations were conducted on several occasions in July and August 1987, in a backyard garden at Remete Kertváros (Budapest, Hungary) and in a commercial apple orchard at Halásztelek, typically from 11 AM until 1 PM.

The dispenser of the attractant consisted of a small piece (10 \times 10 mm) of filter paper attached to a small piece of metal wire. Compounds to be tested were applied in 10- μ l hexane solutions to the filter paper on the spot, a few minutes before starting the observations. Observations with such a dispenser were carried out during ca. 30 min, then both the dispenser and the platform holding it were discarded. For observations with a new dispenser, new platforms were used also.

Observations started when a male could be seen approaching at 50–70 cm from the platform. The males were scored for locating the attractant source and displaying bursts of wing buzzing. The number of visits to the source per male was also recorded. Time spent from landing on the plastic platform to locating the source, spent on the source per male, and spent on the source per visit was also measured.

RESULTS

Trapping. In trials of pure stereoisomers of 5,9-dimethylheptadecane as single attractant compounds at both sites, the *S,S* isomer caught an outstanding number of males (Figure 1). Catches of the other stereoisomers did not signif-

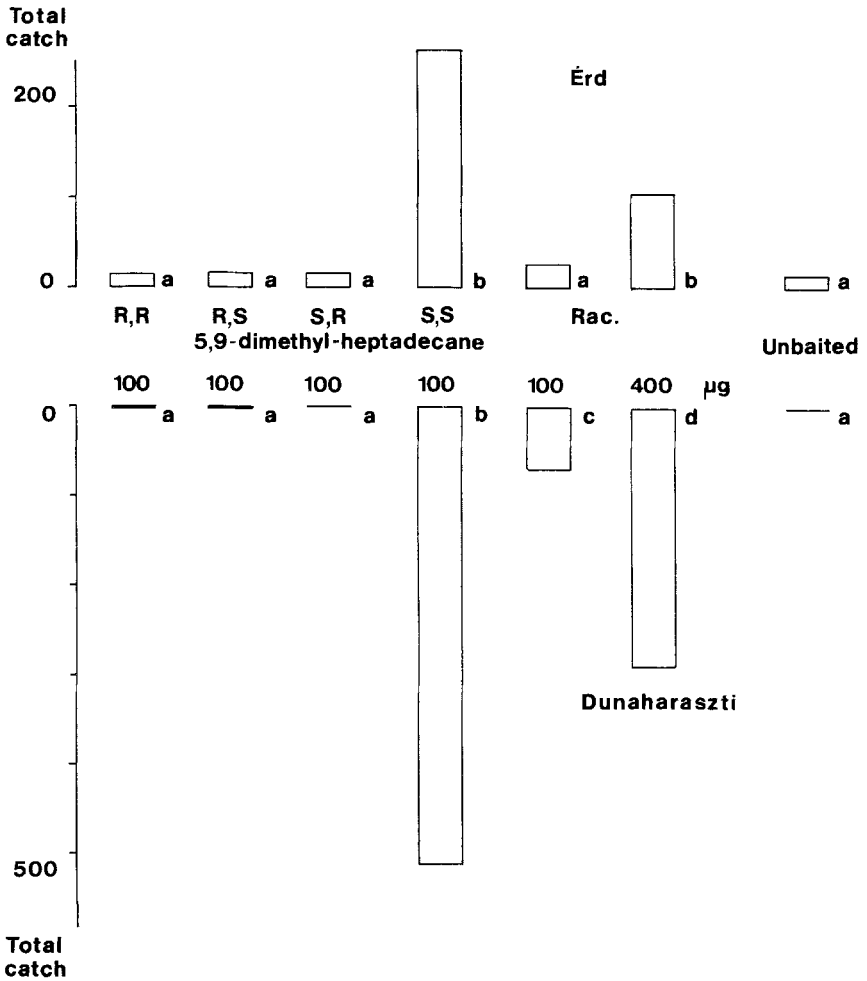


FIG. 1. Captures of male *L. scitella* in traps baited with pure stereoisomers or a racemic mixture of 5,9-dimethylheptadecanes, and unbaited traps. Catches with same letters are not significantly different at $P = 5\%$ by Duncan's NMRT after $\log(x + 1)$ transformation of data. Érd: May 8–19, 1987; four replicates. Dunaharaszti: May 9–21, 1987; four replicates.

icantly differ from the unbaited control. The 100-µg dose of the racemic mixture caught significantly more males than the control only at the site having a higher population density (Dunaharaszti). The catch of the 400-µg dose of the racemic mixture was higher at Érd, not differing from that of the *S,S* isomer.

Low percentages of the racemic mixture of 5,9-dimethylhexadecanes added to the *S,S* isomer did not alter catches (Figure 2) at either site.

Binary mixtures of the *S,S* isomer with the other stereoisomers (ratio: 1 : 1) also caught as many males as the *S,S* isomer alone (Table 1).

In a dosage test, the best catches were obtained with the highest dose of the *S,S* isomer at both the low and high population density sites (Érd, and Halásztelek, respectively) (Figure 3). Lower doses caught gradually decreasing numbers.

No other moth species was attracted to any of the baits in these tests.

Direct Observations of Male Behavior. When setting up a new attractant source, males approached after a few minutes. They moved inside the canopy of trees and, instead of flying freely, flew from one leaf to another in short spurts (not covering more than ca. 15–20 cm on any one occasion) and spent considerable time walking on the leaves or branches before flying further. On most occasions, arriving at the edge or outside corner, they turned against the wind, and remained standing, spreading and slightly moving their antennae before flying again. As they got closer, the males alighted on the platform hold-

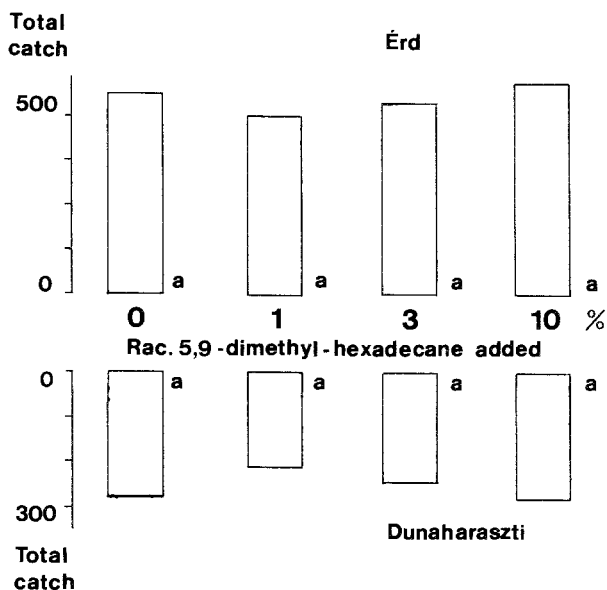


FIG. 2. Captures of male *L. scitella* in traps baited with (*S,S*)-5,9-dimethylheptadecane and its binary mixtures with a racemic mixture of 5,9-dimethylhexadecanes. Catches with same letters are not significantly different at $P = 5\%$ by Duncan's NMRT after log $(x + 1)$ transformation of data. Érd: May 18–June 3, 1987; nine replicates. Dunaharaszti: May 22–June 8, 1987; six replicates.

TABLE 1. CAPTURES OF MALE *L. scitella* IN TRAPS BAITED WITH (*S, S*)-5,9-DIMETHYLHEPTADECANE AND ITS BINARY MIXTURES WITH OTHER OPTICAL ISOMERS^a

5,9-Dimethylheptadecane bait (μg)				Total catch
<i>R, R</i>	<i>R, S</i>	<i>S, R</i>	<i>S, S</i>	
			100	491a ^b
100			100	603a
	100		100	720a
		100	100	326a

^aDunaharaszti, August 14–September 3, 1987; five replicates.

^bCaptures with same letter are not significantly different at $P = 5\%$ by Duncan's NMRT after $\log(x + 1)$ transformation of data.

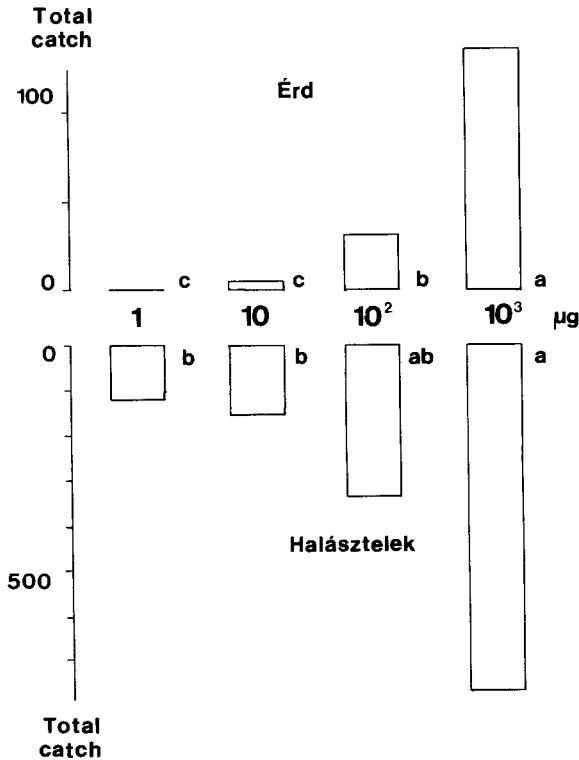


FIG. 3. Captures of male *L. scitella* in traps baited with different doses of (*S,S*)-5,9-dimethylheptadecane. Catches with same letters are not significantly different at $P = 5\%$ by Duncan's NMRT after $\log(x + 1)$ transformation of data. Érd: July 19–25, 1987; three replicates. Halásztelek: August 14–26, 1987; three replicates.

ing the attractant source, at a distance of several centimeters from the source. Once on the platform, they searched for the attractant source exclusively by walking; in no case was the characteristic zigzag flight approach to the source (common to many other moths) observed.

In some cases, males walking on the platform displayed a short burst of wing buzzing, characteristic of the courtship sequence of this species (a detailed description of courtship behavior of *L. scitella* will be published elsewhere).

Arriving at close proximity (less than 1 cm) to the source, these bursts of wing buzzing repeatedly followed each other, and the male climbed up to the filter paper and spent considerable time walking and searching on it. In most cases during this time, it also displayed numerous bursts of wing buzzing.

On several occasions males left the filter paper, walked down to the platform, and then returned for more visits. After one or more visits to the source, males left, in most cases by flying up from the filter paper, and disappearing among the nearby leaves.

In the observations, males responded similarly to both (*S,S*)-5,9-dimethylheptadecane alone and to its mixture with racemic 5,9-dimethylhexadecanes (3%).

Responses to the two attractants did not show significant differences in percentages locating the source, percentages of males displaying bursts of wing buzzing, and number of visits to the source per male (Table 2). Average time spent from landing on the platform to source location was also very similar. This was also true for time spent on the source per male, or per visit.

TABLE 2. RESPONSES OF *L. scitella* MALES APPROACHING ATTRACTANT SOURCES WITH SYNTHETIC PHEROMONE COMPONENTS IN THE FIELD^a

Source (total dose 1 μ g)	Males locating source (%)	Males with burst(s) of wing-buzzing (%)	Visits to source per male	Time (sec) spent		
				From landing on platform to locating source	On source per male	On source per visit
(<i>S, S</i>)-5,9-Dimethyl- heptadecane	71a (<i>N</i> = 24)	71a (<i>N</i> = 24)	1.9a (<i>N</i> = 17)	37.3a (<i>N</i> = 18)	76.9a (<i>N</i> = 18)	37.8a (<i>N</i> = 36)
(<i>S, S</i>)-5,9-Dimethyl- heptadecane + rac- 5,9-dimethyl- hexadecane (3%)	71a (<i>N</i> = 14)	64a (<i>N</i> = 14)	1.3a (<i>N</i> = 10)	34.5a (<i>N</i> = 10)	57.7a (<i>N</i> = 10)	43.6a (<i>N</i> = 13)

^aData with same letters within a column do not significantly differ at $P = 5\%$ (χ^2 test in % locating source and % with wing buzzing bursts; elsewhere Student's *t* test) (In "visits to source per male," only males that did contact the source were evaluated).

DISCUSSION

The results obtained in the trapping tests clearly substantiate that the *S,S* isomer was behaviorally active. Furthermore, other stereoisomers did not synergize or inhibit catches with the *S,S* isomer. This coincides with the fact that catches of a four times higher dosage of the racemic mixture of 5,9-dimethylheptadecanes were close to the level of catch of the pure *S,S* isomer.

On the basis of the above, we conclude that, although behavioral activity is carried exclusively by the *S,S* isomer, a racemic mixture of 5,9-dimethylheptadecanes would compose a usable constituent in practical baits for monitoring *L. scitella*. Since in the dosage test of the pure *S,S* isomer no saturation level was reached, we recommend using higher (several milligrams) doses of the racemic mixture for practical purposes.

Among taxonomically related Lepidoptera, the only known sex pheromone has been identified as 14-methyl-1-octadecene for *Lyonetia clerkella* L. (Sugie et al., 1984). In later studies of this species, only the *S* isomer possessed field activity (Sato et al., 1985). Similar to the case of *L. scitella* in this study, the other enantiomer did not influence catches of *L. clerkella*, and the racemic mixture could be used for practical purposes (Sato et al., 1985).

No increase in trap captures was observed when small percentages of racemic 5,9-dimethylhexadecane, which was found previously to be present in pheromone effluvia of females (Francke et al., 1989), were added to (*S,S*)-5,9-dimethylheptadecane.

Also, there was no outstanding difference in the behavior of males approaching an attractant source containing a mixture of the heptadecane and hexadecane vs. the heptadecane alone. Consequently, in this study we failed to show any behavioral activity for the previously identified minor component 5,9-dimethylhexadecane. It is possible, however, that this compound influences behavioral events not studied here or other aspects of reproductive behavior.

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PREFERENCE FOR MAGNESIUM SULFATE-TREATED
LEGUMINOUS SEEDS IN EGG-LAYING BEAN
WEEVIL (*Acanthoscelides obtectus* Say, Col.,
Bruchidae)

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Abstract—Unlike many secondary plant substances, a wide range of concentrations (4–1000 mM) of magnesium sulfate, applied to dry beans, significantly increased egg-laying by the dry bean weevil in binary choice tests, in favor of treated seeds. No other magnesium-containing compounds studied exerted such an effect, nor was a similar response noted on treated beans in no-choice situations. The total number of eggs laid per female was in the same range in both types of test. Variably enhanced or suppressed oviposition responses were shown on magnesium sulfate-treated secondary hosts and on nonhosts or on indifferent substrates. No specific behavior by egg-laying bean weevil females on Mg-treated seeds could be detected. The results are explained by assuming the functioning of magnesium as a supernormal stimulus for egg-laying. However, a physiological effect on neuromuscular synaptic transmission, as a consequence of probable Mg uptake resulting in a decreased propensity to move, is also hypothesized.

Key Words—Magnesium sulfate, oviposition, bean weevil, *Acanthoscelides obtectus* Say, Coleoptera, Bruchidae, supernormal stimulus, neuromuscular synaptic depression.

INTRODUCTION

In many biochemical and physiological processes of various species, including man, the presence of magnesium ions is considered to be indispensable (Aikawa, 1981; Ferment and Touitou, 1985). As for insects, water-soluble magnesium compounds usually are deterrent (Dethier, 1968; Simpson, 1978). However, a

strong preference was shown by egg-laying females of egg parasitoids (*Trichogramma* spp.) for artificial host eggs containing higher or close to host hemolymph levels of magnesium and potassium acting synergistically (Nettles et al., 1982, 1983).

In the presence of plant allelochemicals and of inorganic substances applied to dry bean seeds, egg-laying by the bean weevil, *Acanthoscelides obtectus* Say (Coleoptera, Bruchidae), has been depressed to various extents (Muschinek et al., 1976; Jermy and Szentesi, 1978; Szentesi, 1983). Among the substances examined to date, only magnesium sulfate showed the opposite effect, i.e., egg-laying was enhanced by magnesium-treated beans as compared to untreated ones (Jermy and Szentesi, 1978). To our knowledge, no other data exist in the literature concerning the role of magnesium in influencing oviposition in phytophagous insects.

Jermy and Szentesi (1978) briefly indicated the preference showed by the bean weevil for magnesium-treated beans; it was the aim of the present paper to investigate the nature and extent of this preference, examining: (1) the range of compounds eliciting a similar response, (2) the stimulus situation in which the preference is manifested, and (3) whether preference is accompanied by specific behavioral patterns. There is also discussion of two possible modes of action: (1) whether magnesium-treated beans represent a supernormal stimulus, and (2) whether possible uptake of magnesium sulfate might result in changes of behavior reflecting a neurophysiological effect.

METHODS AND MATERIALS

The dry bean weevils were obtained from a mixture of wild (heterogeneous) populations maintained in the laboratory for at least three years on dry beans (Szentesi, 1972). Freshly emerged adults were sieved, kept in glass jars lined with corrugated paper strips, and supplied with 10% honey-water. Adults mated in the jar and were used at the age of 4 days.

Oviposition was measured in choice and no-choice tests on treated and untreated beans. The surface of the seeds was coated with a given compound by a method described by Muschinek et al. (1976). In short, 0.25 ml of a compound's solution or suspension was dried onto the surface of 10 g seeds by a cold or warm (about 93–96°C) airstream for 1 min.

The quantity of magnesium salts, both administered and naturally present, was determined by chemical analysis and by atomic absorption spectrophotometry (AAS). The results are given as percentage magnesium in relation to absolute dry matter (Table 1).

The uniformity of magnesium sulfate deposits on coated surfaces was assessed on 8-mm-diam. (201-mm² surface) glass beads, treated by the standard

TABLE 1. MAGNESIUM CONTENT OF LEGUMINOUS SEEDS AND PLANT PARTS AND MAGNESIUM SULFATE-TREATED SUBSTRATES TESTED FOR OVIPOSITION (AAS ANALYSIS)

Substance	Percentage of magnesium content ^a
Beans ^b	0.170, 0.13 ^c
Soybeans ^b	0.289, 0.27 ^d
Peas ^b	0.147, 0.15 ^e
Lentil ^b	0.127
Bean varieties (<i>N</i> = 4)	0.185-0.214
Bean seeds treated with 111 mM or 3000 mM magnesium sulfate	0.198
Magnesium sulfate (111 mM) treatment for	
1 min	0.211
5 min	0.208
10 min	0.211
Bean seed cotyledon	0.146
Bean seed testa	0.447
Bean pods	0.427
Bean pod stem	0.413

^aRefers to absolute dry matter content.

^bDry whole seeds.

^cNehring et al. (1972).

^dMándy et al. (1980).

^eRaboy et al. (1984).

procedure with 37 mM magnesium sulfate. Microscopic examination has shown that there were 7.1 ± 0.7 (mean \pm SE) magnesium sulfate crystals per square millimeter on the glass beads. The size of the crystals varied between 0.04 and 0.87 mm.

Titrimetry showed that the loss of chemicals during treatment was within the range of 5-10% and that individual seeds received, e.g., in case of 111 mM concentration, 0.103 mg magnesium sulfate per square millimeter of seed surface.

Magnesium sulfate was incorporated into pills. Pills of different kinds consisting of bean pod or seed powder, potato starch, and cellulose powder were prepared in various compositions. As sticking materials, distilled water, glycerine and soluble cellulose, to which magnesium sulfate was added to give a 100 mM concentration, were used. The 6-mm-diam. pills were prepared with a pharmaceutical device. These were dried at 45°C and used for oviposition tests. The pills of different types weighed 91 ± 2 (SD) to 165 ± 7 mg.

In choice tests, solvent-treated and coated leguminous seeds were offered

in 10-cm-diam. Petri dishes divided into four sections. Opposite sections contained the same stimulus. Ten males and 10 females were confined in each dish for 10 days. For no-choice tests, 2.8×5 -cm size vials were used, each having three or four pieces of oviposition material and three to four males and females. The vials were covered with pieces of linen cloth.

Tests were conducted in total darkness, at 23°C and 60–70% relative humidity, without food or water for the adults. The number of eggs laid per female was used for evaluation. Preference was expressed by a discrimination quotient (DQ) (David and van Herrewage, 1970) (see legend of Figure 1). Statistical evaluations were done on the basis of the number of eggs laid [Wilcoxon's signed-ranks test, Student's t test, or Duncan's new multiple-range (DNMR) test; Steel and Torrie, 1960; Sokal and Rohlf, 1969].

All chemicals used were of analytical grade, unless otherwise stated.

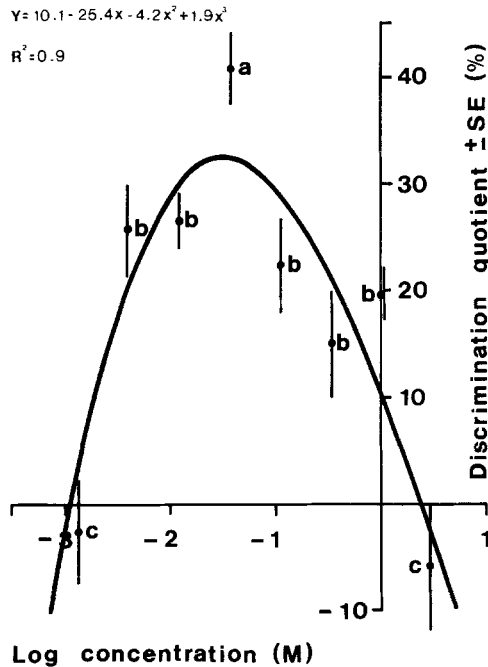


FIG. 1. Dose-response curve of egg-laying *A. obtectus* females to beans in binary choice tests treated with magnesium sulfate at different concentrations. Values are expressed as discrimination quotient (DQ) (David and van Herrewage, 1970) given by the ratio: (No. of eggs laid on treated - No. of eggs laid on control)/Total No. of eggs laid. To obtain a percentage value, the ratio is multiplied by 100. Concentrations: 1.3, 4, 12, 37, 111, 333, 1000, and 3000 mM. SE = standard error (bars). Means followed by the same letters are not significantly different at the 1% probability level (DNMR test for ESS).

Experiments with Magnesium Sulfate on Bean Seeds. Eight millimolar levels of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were examined from 1.3 to 3000 (Figure 1). Magnesium sulfate of two purity grades, different lengths of treatment time, cold and warm application, as well as different exposure times of treated seeds to ovipositing adults were also tested.

Experiments with Other Magnesium or Sulfate Compounds. In addition, other magnesium compounds (Table 3) were tested at 111 mM. Some of them were applied as suspensions in 2% potato starch solution, while aqueous solutions were used for the rest. Other sulfate-containing compounds and sulfuric acid were also tried (Table 3).

Experiments with Magnesium Sulfate on Various Oviposition Substrates. Heterogeneous-type bean weevil females lay eggs on a range of substrates (Pouzat, 1975; Basky, 1977; Szentesi, 1976; Jermy and Szentesi, 1978). Occasional and secondary hosts (peas, lentils, and soybeans), and partially or totally indifferent substrates (e.g., glass beads, starch + cellulose pills, dry seed cotyledon devoid of testa) were also treated with magnesium sulfate (111 mM conc.). These substrates were offered both in choice and no-choice tests for egg-laying.

Experiments on Direct Effect of Magnesium Sulfate on Adult Behavior. Twenty 2- to 4-day-old *A. obtectus* females deprived of egg-laying but mated were allowed to oviposit on control and 111 mM Magnesium sulfate-treated seeds. Single seeds with hilum upright were stuck to the bottom of 2.8×5 -cm glass vials. Homogeneous illumination (1900 lux) was provided. Temperature inside the open vial was 30°C . Individual mated females were put onto the beans and allowed to perform preovipositional behavior, which was observed through a binocular microscope and simultaneously tape-recorded.

Bean weevil adults can take up magnesium sulfate from treated seed surfaces, and this might affect behavior. For "voluntary" uptake, 3-day-old mated females were allowed to move and lay eggs on untreated or treated seeds, among which cotton wicks were placed containing 1 ml honey-water or honey-water + 10 or 100 mg magnesium sulfate. The cotton wicks were replaced daily (five females per Petri dish; 11 replicates). For "forced" uptake of magnesium sulfate two groups of adults were confined in cages without beans for three days. The cages were kept at 28°C , and drinking of honey-water containing the salt (100 mg/ml) was ensured. "Magnesium drunk" adults (10 males and 10 females) were transferred onto dry beans (15 replicates). In both cases locomotor activity of adults was monitored for 3-4 hr daily for four to nine days, at 15-min intervals. The eggs laid were also counted.

In a choice test, presence of a mated female in the treated or untreated section was recorded hourly, 3-8 hr/day, for 13 days. Light intensity was 600 ± 300 lux (299 replicates).

The effect of "built-in" magnesium on moving activity was examined by rearing the bean weevils both in control pills containing 0.5% magnesium sul-

fate (near the range occurring in whole seeds of legumes: Nehring et al., 1972, cited by Szabó, 1980; Spector, 1956, cited by Mándy et al., 1980), and in treated ones containing 1, 2.5, and 5% of magnesium sulfate. Pills were infested with a single egg each. The adults that emerged were put onto untreated bean seeds in Petri dishes. Ambulatory movement of six adults (three females and three males) per replicate was recorded for nine days for 4–8 hr/day at half-hour intervals (seven to nine replicates).

RESULTS

AAS analysis revealed that bean seed coat, bean pod, and bean pod stem contained approximately two to three times more magnesium than whole seeds of some bean varieties or than bean cotyledon alone. Leguminous seeds were in the same range as dry beans, while soybeans contained a slightly higher percentage (Table 1).

Effects of Bean Seeds Covered with Magnesium Sulfate. In choice tests beans treated with 4–330 mM magnesium sulfate were significantly preferred for egg-laying with a maximum at 37 mM (Figure 1). In no-choice tests, the number of eggs laid with a concentration spectrum of 4–3000 mM ranged between 37 ± 7 (mean \pm SD) and 44 ± 8 , and none of the values was significantly different. On control beans, females laid 37 ± 8 eggs. Also, beans were equally preferred whether treatment was applied warm or cold (Table 2). Preference for treated seeds gradually decreased as treatment time increased. Impurities in the compound (e.g., other unknown inorganic elements) or ways of treatment did not influence preference (Table 3).

Effects of Other Magnesium or Sulfate Compounds. No bean seeds treated with other magnesium or sulfate-containing compounds were preferred in choice tests, although some proved to be neutral. The sulfate moiety alone slightly depressed egg-laying (Table 3).

Effects of Magnesium Sulfate Administered to Various Oviposition Substrates. Generally, preference for other magnesium sulfate-treated leguminous seeds and for treated bean seed coat pills increased (Table 4). In spite of the magnesium treatment, indifferent substrates (e.g., glass beads, starch pills) were not preferred. Females laid significantly ($P = 0.5\%$) more eggs on peeled halved bean cotyledons treated with magnesium sulfate (Table 4).

Results of Behavioral Experiments. No specific behavior related to magnesium sulfate treatment was observed. The exploratory phase comprising palpation, chewing into and around the hilum, etc., as well as preoviposition and egg deposition phases, all seemed to be performed in the same manner on magnesium-treated and control beans.

Free access to 10 or 100 mg magnesium sulfate/ml honey–water did not result in a decrease of moving activity during the nine days of observation (Table 5).

TABLE 2. EGG-LAYING RESPONSE OF *A. obtectus* FEMALES TO EXPOSURES AND TREATMENTS OF 111 mM MAGNESIUM SULFATE ON DRY BEANS IN BINARY CHOICE TESTS ($N = 9-14$)

Treatments and exposure	DQ ^a (% ± SD)	Level of significance (%)
Treatments		
Warm airstream	+32 ± 12a ^b	0.5 ^c
Cold airstream	+36 ± 18a	0.5
Duration of treatment		
1 min	+25 ± 17A	0.5
5 min	+4 ± 17B	NS
10 min	-8 ± 16B	NS
Exposure to treated seeds for		
2 days	+30 ± 16a	0.5
4 days	+28 ± 10a	0.5
6 days	+37 ± 13a	0.5
8 days	+32 ± 13a	0.5
10 days	+30 ± 19a	0.5

^aDiscrimination quotient (David and van Herrewage, 1970).

^bMeans in the column followed by the same lower- or uppercase letters are not significantly different at the 1% probability level (DNMR test for ESS).

^cDenotes statistical comparison with own control and *not* within column (Wilcoxon's signed-ranks test by paired values of eggs laid).

TABLE 3. EGG-LAYING RESPONSES BY *A. obtectus* FEMALES TO MAGNESIUM COMPOUNDS, IMPURITY LEVELS OF MAGNESIUM SULFATE, AND INORGANIC SULFATES ON DRY BEANS IN BINARY CHOICE TESTS (CONC. 111 mM, $N = 9-19$)

Compounds	DQ ^a (% ± SD)	Level of significance (%)
Magnesium compounds		
Sulfate	+22 ± 14	0.5 ^b
Hydroxide	0 ± 15	NS
Fluoride	-6 ± 13	0.5
Chloride	-14 ± 11	0.5
Nitrate	-46 ± 15	0.5
Citrate	-34 ± 15	0.5
Impurities of magnesium sulfate		
analytical vs. technical grade	+5 ± 17	NS
Inorganic sulfates		
Sulfuric acid (pH 5.5)	-14 ± 13	0.5
Nickel	-2 ± 20	NS
Zinc	-36 ± 14	1.0
Barium	-19 ± 19	2.5

^aSee Table 2 for explanation.

^bDenotes statistical comparison with the own control, but *not* within column (Wilcoxon's signed-rank test calculated by the paired values of eggs laid).

TABLE 4. EGG-LAYING RESPONSES OF *A. obtectus* FEMALES TO MAGNESIUM SULFATE-TREATED SUBSTRATES IN BINARY CHOICE TESTS (CONC. 111 mM, *N* = 7-10)

Oviposition substrates	DQ ^a (% ± SD)	Level of significance (%)
Leguminous seeds + magnesium sulfate		
Dry beans	+22 ± 14	0.5 ^b
Lentils	+33 ± 20	1.0
Peas	+20 ± 28	5.0
Soybeans	+6 ± 14	NS
Bean cotyledon (no testa)	+43 ± 15	0.5
Host constituents in pills + magnesium sulfate		
Bean seed coat	+38 ± 31	1.0
Bean pod	-11 ± 25	NS
Indifferent substrates + magnesium sulfate		
Starch pilules	-34 ± 12	2.5
Glass beads	-5 ± 53	NS

^a See Table 2 for explanation.

^b Denotes statistical comparisons with the own control, but *not* within column (Wilcoxon's signed-ranks test calculated by the paired values of eggs laid).

TABLE 5. MOVING ACTIVITY OF *A. obtectus* ADULTS AFTER VOLUNTARY AND FORCED UPTAKE OF MAGNESIUM SULFATE FROM DRINKING WATER (HONEY-WATER) (*N* = 11-15)

Solution	Moving activity (mean ± SD) per female per 15 min (observational units)	
	Free access	Forced uptake
10 mg magnesium/ml (untreated beans)	0.11 ± 0.09a	—
100 mg magnesium/ml (untreated beans)	0.09 ± 0.07a	0.11 ± 0.06A
Honey-water only (magnesium-treated beans)	0.12 ± 0.09a	—
Honey-water only (untreated beans)	0.11 ± 0.8a	0.19 ± 0.05B

a = NS; A and B = $P \ll 0.1\%$ (Student's *t* test, $t_{\text{calc.}} = 8.149$, $df = 118$).

The rate and amount of magnesium uptake by the females is unknown, although they were observed drinking honey-water containing the salt. Mortality was the same for all groups during the observation period.

“Forced” uptake of magnesium significantly decreased moving activity (Table 5). The number of eggs laid by the two groups were: 75.3 ± 2.5 (control) vs. 33.2 ± 6.1 (“magnesium-drunk”) (mean \pm SD) eggs per female ($P < 0.1\%$, Student’s t test, $t_{\text{calc.}} = 14.08$, $df = 28$). High mortality ($37.3 \pm 11.8\%$ vs. $3.0 \pm 4.1\%$) occurred among “magnesium-drunk” adults by the third day of observation.

Mated females stayed significantly more frequently on magnesium-treated beans in choice tests. During 13 days, 200 females were found on 12,500 occasions on, under, or in the immediate vicinity of magnesium-treated beans vs. 7994 occasions on untreated ones ($P = 1\%$, Wilcoxon’s signed-ranks test for paired observations). The egg-laying response reflected the same preference for magnesium-treated seeds [32.4 ± 14.7 (mean \pm SD) vs. 26.6 ± 14.2 eggs, $P = 0.1\%$, $t_{\text{calc.}} = 3.43$, $N = 200$].

Adults that emerged from pills containing elevated magnesium during their larval development did not show any decreased level of moving activity following emergence.

DISCUSSION

Phytophagous insects live in a chemically extremely diverse milieu where a multitude of plant chemicals carry information. The principal components of this milieu are organic molecules. In contrast, inorganic substances are only very rarely the basic determinants of acceptance or rejection, although some insects do show characteristic responses to them (Dethier, 1968; Simpson, 1978). Earlier studies cited by Dethier (1953) on taste and oviposition perception of electrolytes by several species of insects, listed magnesium ions among the unacceptable cations, albeit not among the most unacceptable ones.

The experiments carried out in this study not only strengthened the earlier finding (Jermy and Szentesi, 1978), namely, that ovipositing females preferred magnesium-treated bean seeds, but they also revealed that a relatively wide (circa 100-fold) concentration range (4–330 mM) of magnesium sulfate “stimulated” egg-laying (Figure 1). Such direct stimulation by magnesium and other cations acting synergistically has been shown so far only in an egg-parasitoid (Nettles et al., 1982, 1983). Thus, this is the only known case of magnesium sulfate by itself enhancing oviposition of a phytophagous insect species.

The AAS analysis revealed that the distribution of magnesium is more or less specific in the host plant (Table 1). The results of whole seed analyses agree with data in the literature (see references in Table 1). Some plant parts

(seed testa, pod, and stem) contain approximately twice as much magnesium as the whole seed and three times more than seed cotyledon alone. As bean weevil females come into contact with the seed coat during oviposition, the pronounced preference for the seed could be partially explained by the presence of this single influential factor, although other chemicals acting on the olfactory and gustatory systems, as well as shape or size, etc., of the oviposition substrate are also important (Avidov, 1965; Pouzat, 1974a,b, 1975; Szentesi, 1976).

As for the perception of magnesium deposits on the treated seeds, Chapman (1977) has shown that insect gustatory receptors can respond to solid substances. Uptake of magnesium deposits from the bean surface is likely to occur, since ejection and uptake of small quantities of saliva by the bean weevil, while examining the seed surface, can regularly be observed (Huignard, personal communication; Szentesi, unpublished). That magnesium ions might be able to bond to substances on the surface of dry seeds during surface treatment seems improbable. Binocular (and scanning electron) microscopic examinations have revealed no conspicuous changes on the surface of the treated seeds. A chemical alteration of the seed surface, although possible, is unlikely.

Treatment time did alter the oviposition response, namely, the longer the coating process took the less acceptable the seed became (Table 2). No explanation is suggested for this phenomenon. (Usual treatment time never exceeded 1 min.) The biased egg-laying response had occurred by two days and did not show a further change from then on (Table 2).

Other magnesium- or sulfate-containing compounds showed either oviposition inhibition, namely, the water-soluble ones, or were ineffective (the weakly or not soluble compounds) (Table 3). The anion moiety alone could not be responsible for the preference for magnesium sulfate, since sulfuric acid treatment and other sulfate-containing chemicals did not increase the acceptability of bean seeds (Table 3).

The females responded positively not only to treated dry beans, but also to other leguminous seeds following magnesium treatment. For instance, the ovipositional status of lentil, pea, and bean cotyledon devoid of testa, and pills incorporating bean seed coat improved relative to their own respective controls, although their suitability for egg-laying without magnesium treatment was relatively low (Table 4). On the other hand, although soybeans were relatively highly rated for oviposition by *A. obtectus* females (Jermy and Szentesi, 1978), magnesium sulfate treatment decreased this status. Starch pills, a poor egg-laying substrate were even inferior after magnesium treatment, while glass beads remained neutral in effect.

Direct observation of egg-laying females on magnesium sulfate-treated beans did not reveal any specific behavioral pattern related to the presence of

this salt. However, an arrestant effect of magnesium sulfate was proven in choice tests in which females were found significantly more frequently staying on treated beans, resulting also in a significantly higher number of eggs laid on such seeds. This arrestment effect seems to support the notion that magnesium-treated bean seeds may function as a supernormal stimulus for the bean weevil females, as defined by Manning (1972). The natural magnesium content of seed testa, acting together with factors such as shape, specific organic substances, etc., provide the normal stimulus pattern, while an additional 20–40% increase of magnesium by treatment with the salt represents supernormality. As a result, in choice tests magnesium-treated seeds were more readily oviposited upon (Figure 1), while in a no-choice situation there was always the same number of eggs laid on both types of seeds. The latter merely refers to the maximum response possible and that magnesium sulfate alone does not stimulate egg-laying.

Supernormality presupposes a stimulus situation in which choice can be exercised, or in which essential stimuli (shape, size, coloration, etc.) are strongly emphasized (McFarland, 1981). Although, such exaggerated behavioral responses are mostly documented in insects to visual stimuli (Prokopy, 1969; Magnus, 1958), the case reported here may indicate the effect of a supernormal chemical stimulus.

The above reasoning does not exclude another explanation on physiological grounds considering that, during thorough palpation and “licking” of the treated surface, some magnesium sulfate must be ingested. Considering the many important roles of magnesium in a living organism (Bara, 1977; Aikawa, 1981; Levine and Coburn, 1984; Ferment and Touitou, 1985), it has been shown that excess magnesium ions might depress both neuromuscular and other types of synaptic transmission, as shown in insects by Hoyle (1955), Callec (1974), Fukami et al. (1984); in mollusks by Burton et al. (1987); and in vertebrates by del Castillo and Engbaek (1954), Ault et al. (1980), Artemenko and Gerasimov (1984), and others. The depression would then result in a low propensity to move. However, an observable reduction in moving activity was found only when adults of *A. obtectus* were forced to take up magnesium sulfate with drinking water (Table 5) and not in the case of free access to magnesium-containing drinking water or when having developed in pills with elevated salt concentration. Nevertheless, it cannot be excluded that a very slight decrease in propensity to move, which may easily escape direct observation, may result in longer stays on treated beans, i.e., in increased number of eggs laid on them. This is the more likely since the behaviorally active doses that were applied in the study were well within the range found effective in other studies (e.g., Nettles et al., 1982, 1983; del Castillo and Engbaek, 1954).

CONCLUSIONS

The preference of egg-laying by *A. obtectus* females for magnesium sulfate-treated beans can be explained partly by the two hypotheses, although the functioning of magnesium sulfate as a supernormal chemical stimulus seems more probable. It is also likely that females do not find a situation in nature where such a preference could be exercised; therefore, the adaptive significance of the behavior is highly questionable. However, a possible implication would be that an increase of magnesium concentration in the hemolymph or blood of hibernating or overwintering animals provides a means of freeze-resistance (Aikawa, 1981). Magnesium uptake by bean weevil adults could serve a similar purpose as they also hibernate as adults. However, both hypotheses and, especially, the latter speculation would need further investigation to clarify the details sufficiently.

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FEMALE SEX PHEROMONE OF IRIS BORER,
Macronoctua onusta (LEPIDOPTERA:
NOCTUIDAE)

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Abstract—Chromatographic and mass spectrometry studies of heptane extracts of the ovipositors of the iris borer, *Macronoctua onusta*, showed that the females produce several compounds that are the same as those produced by females of the tobacco budworm, *Heliothis virescens*. In trapping experiments, a mixture of (Z)-11-hexadecenal, (Z)-11-hexadecen-1-ol, and (Z)-9-tetradecenal (94:4:2) proved to be the minimum set of compounds required to cause effective capture of iris borer males in the field.

Key Words—(Z)-11-Hexadecenal, (Z)-11-hexadecen-1-ol, (Z)-9-tetradecenal, iris, iris borer, *Macronoctua onusta*, tobacco budworm, *Heliothis virescens*, Lepidoptera, Noctuidae.

INTRODUCTION

The iris borer (*Macronoctua onusta*, Grote) is the major lepidopterous pest insect in iris (*Iris versicolor* L.) throughout much of North America. Larvae, feeding in rhizomes, can destroy entire iris beds. Adults of the species are nocturnal and they emerge, mate, and females lay eggs on iris in the fall (September–October). Eggs hatch in spring, and larvae feed in the leaves and then bore into the rhizomes (Schread, 1970; Neiswander, 1961). We became interested in determining the composition of the female sex pheromone of this insect because it was thought that a synthetic replica of the pheromone might prove useful to iris growers wishing to monitor flight activity of the pest.

We report that the array of pheromonal compounds produced by the iris borer female resembles those previously found in the female tobacco budworm, *Heliothis virescens* (Fabricius) (Klun et al., 1980; Teal et al., 1986), and that a mixture of *Z*-11-hexadecenal, (*Z*)-11-hexadecen-1-ol and (*Z*)-9-tetradecenal (94:4:2) is the minimum set of compounds required to effectively cause capture of iris borer males in the field.

METHODS AND MATERIALS

Insects. In August 1983 and 1984, rhizomes infested with late-instar larvae of the borer were collected from iris beds in the vicinity of Beltsville, Maryland, transplanted to potting soil, and held in a greenhouse. Pupae of the borer were recovered from the pots and transferred to screen-covered plastic containers containing ca. 5 cm moist soil to await emergence of adults. Adult females were held at ambient conditions of the greenhouse for two to three days after emergence. At that time, their ovipositors were excised at the onset of scotophase, soaked in ca. 25 μ l heptane/ovipositor for ca. 10 min, and then the solvent was drawn away from the ovipositors and stored at -4°C in a screw-cap vial. In one case, a set of six ovipositors was extracted twice: first by a brief soak (10 min) in 100 μ l heptane followed by soaking in a second volume of solvent for 1 hr. The onset of scotophase was selected as the time for preparation of extracts because previous work (Schread, 1970) indicated that the adults mate nocturnally, and we surmised that pheromone titers in the females would likely be highest at that time. In all, we obtained extracts of four females in 1983 and 10 females in 1984 that permitted analyses by capillary gas chromatography (GC), using polar and nonpolar columns (Klun and Huettel, 1989), and also analysis by combined GC-mass spectrometry (MS), using instruments and operating conditions described by Klun et al. (1982).

Attempts to obtain additional adult females by rearing early-instar field-collected larvae on artificial diets of the European corn borer (Reed et al., 1972) and corn earworm (Burton, 1970) were unsuccessful. Consequently, supplies of insects for chemical and field studies were severely restricted throughout this research and virgin females were available for inclusion in field trapping experiments in only the first year of the study.

Chemicals and Male Trapping Tests. All compounds were previously synthesized at the Insect Chemical Ecology Lab and were purified by preparative liquid chromatography using two 25-cm \times 10-mm (ID) stainless-steel tubes packed with 20% AgNO_3 -impregnated 10 μ m Spherisorb and toluene as eluant. All compounds were geometrically pure and were greater than 95% chemically pure according to GC analyses. In field trapping tests, conducted during October 1984–1987, mixtures of the compounds identified from female ovipositor

extracts were applied in microgram amounts to either cotton dental rolls (1984 season) or rubber septa (Thomas Scientific, catalog No. 1780-J07) that were positioned in Pherocon 1C, or *Heliothis* Scentry (1986 field tests only) insect traps (Great Lakes IPM, Vestaburg, Michigan 48891). The cotton rolls were used initially in the field tests because one of us (J.A.K.) had used them previously in a study of the tobacco budworm pheromone (Klun et al., 1980) and found that, when an appropriate mixture of compounds was evaporated from cotton rolls in field traps, they caused capture of males more effectively than traps baited with four virgin tobacco budworm females. Inasmuch as the compounds we were dealing with were the same as those of the tobacco budworm, we reasoned that cotton was a suitable substrate to use in the iris borer field tests. The cotton rolls were replaced nightly, while fresh septa were positioned in the traps weekly. The septa were chosen as evaporative substrates for the bulk of the field tests for convenience and because they have been used routinely to control the evaporation of compounds in studies of the pheromone systems of many moths (Heath et al., 1986). When Pherocon 1C traps were used, the lower interior surface of the traps was coated with Tack Trap to ensure trap-capture efficiency at the cool nighttime temperatures of October; Tack Trap is a polymer that is less viscous than the adhesive normally used by the commercial manufacturer. Virgin iris borer females were individually hung in traps within a small cylindrical cage. Traps were suspended ca. 1 m from the ground on stakes driven into the ground. The traps, containing specific mixtures of compounds, were positioned in urban areas known to have iris plantings. Traps were ca. 10 m apart, and all tests were replicated over time and were conducted using a randomized complete block design with 1–20 km between blocks (replicates). Duncan's multiple-range test was applied to test for significant differences between mean total male captures across replicates for each treatment.

RESULTS AND DISCUSSION

GC and GC-MS. Figure 1 shows the chromatogram obtained by analysis of an aliquot of sample (ca. 0.2 female equivalent) of the combined ovipositor extracts of four iris borer females in 1983 with a Carbowax 20 M capillary column. This analysis and subsequent analyses of samples taken from other extracts showed that they all contained the nine pheromone-like compounds. The identities of these compounds were first established by coincidence of their retention times with standard compounds on Carbowax 20 M and DB-1 capillary columns, and then all of them were verified by GC-MS; the mass spectra of compounds detected were identical to those of the standard compounds. The percentage composition of the pheromonal compounds in extracts of four females, the combined extracts of six females (rinse and soak), three females,

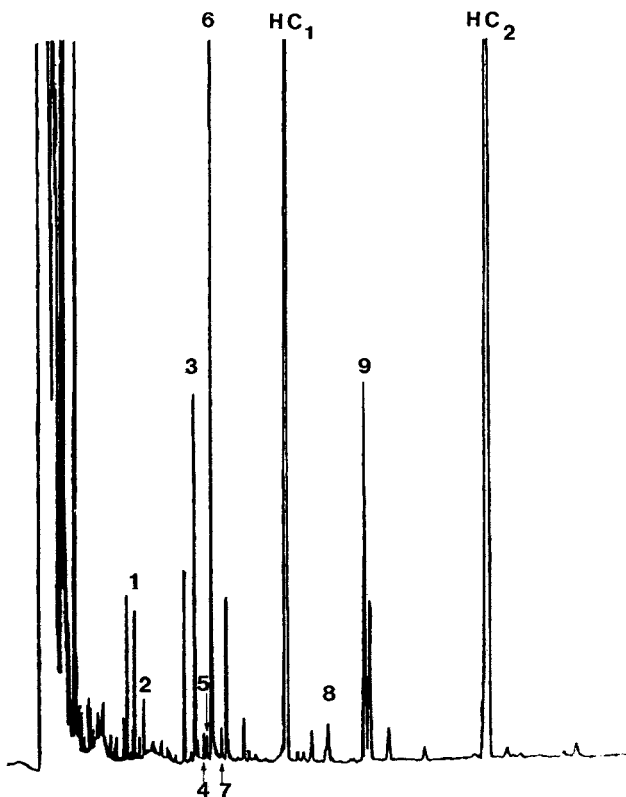


FIG. 1. GC chromatogram (Carbowax 20M capillary) obtained by using a sample of the combined heptane extracts of four iris borer ovipositors. 1 = 14:Ald, 2 = Z9-14:Ald, 3 = 16:Ald, 4 = 14:OH, 5 = Z9-16:Ald, 6 = Z11-16:Ald, 7 = Z9-14:OH, 8 = 16:OH, and 9 = Z11-16:OH. HC₁ = tricosane; HC₂ = pentacosane. Other peaks in the chromatogram were contaminants in the solvent or did not appear regularly from one analysis to another.

and an individual female are shown in Table 1. It is noteworthy that the set of compounds produced by females of the iris borer are similar to those produced by the tobacco budworm, *Heliothis virescens* (Teal et al., 1986); all compounds found in the tobacco budworm, with exception of Z7-16:Ald, occur in the iris borer. The major component in the extracts was (*Z*)-11-hexadecenal (Z11-16:Ald), with exception of a solution obtained by soaking the ovipositors in the solvent for an hour; it contained (*Z*)-11-hexadecen-1-ol (Z11-16:OH) in greatest abundance (57%). Inasmuch as the 10-min rinse of the same ovipositors contained proportionately less alcohol than that obtained by prolonged

TABLE 1. PERCENTAGE COMPOSITIONS OF IRIS BORER OVIPOSITOR EXTRACTS^a

Compound	Percentage composition					
	1983			1984 (DB-1)		
	4 females			6 females		
	DB-1	Carbowax 20 M	Rinse	Soak	3 females	1 female
14: Ald	2.1	2.7	1.4	1.7	1.7	1.8
Z9-14: Ald	0.9	1.3	1.2	2.0	2.4	0.6
16: Ald	11.4	11.0	4.8	2.8	4.5	10.3
14: OH	0.5	0.7	0.2	0.6	0.1	0.1
Z9-16: Ald	0.5	0.7	0.7	1.0	0.7	0.9
Z11-16: Ald	58.9	55.6	65.8	33.2	50.7	59.7
Z9-14: OH	0.8	0.9	0.6	1.7	0.7	0.2
16: OH	1.5	1.6	0.4	0.5	0.7	0.3
Z11-16: OH	23.3	25.3	24.9	56.6	38.4	26.1

^aDB-1 is data from a nonpolar column and Carbowax 20 M is data from a polar column.

extraction, it is probable that the large quantity of alcohol found in the extracts is reflective of a comparatively large alcohol metabolic pool that exists within the pheromone gland. The alcohol in the iris borer is quite likely a biosynthetic precursor of Z11-16: Ald; in *Heliothis*, Tumlinson and Teal (1987) have shown that an alcohol oxidase in the insect cuticle is responsible for generation of pheromonal aldehydes, and it is reasonable to suspect that a similar enzyme occurs in the iris borer. The next most abundant compound in the iris borer extracts was hexadecanal (16: Ald) along with traces of tetradecanal (14: Ald), (Z)-9-tetradecenal (Z9-14: Ald), tetradecan-1-ol (14: OH), (Z)-9-hexadecenal (Z9-16: Ald), (Z)-9-tetradecen-1-ol (Z9-14: OH), and hexadecan-1-ol (16: OH).

In the first field-trapping experiment (1984), three mixtures of compounds and virgin females were deployed (Table 2). Treatment 1 contained all pheromonal compounds identified in the female extracts with exception of the trace amounts of 14: OH and 16: OH; these were excluded to minimize the possible number of permutations of compounds to be tested. The ratio of Z11-16: Ald to Z11-16: OH (73:27) in treatment 1 approximated the ratio observed in ovipositor extracts of 1983. Treatment 2 lacked Z11-16: OH, and treatment 3 differed from treatment 1 in that the Ald-OH ratio was arbitrarily set at 96:4. Proportionately less alcohol was selected for treatment three because we surmised that the ratio observed in the female extracts overestimated the amount of alcohol that the females might actually release when calling for a mate. The

TABLE 2. MIXTURES OF COMPOUNDS TESTED AGAINST IRIS BORER AND MALE CAPTURES IN FIELD TRAPS^a

Compound (μg)/dispenser	Treatment									
	1	2	3	0	4	5	6	7	8	9
Cotton dispenser										
2.8 14:Ald	X	X	X							
2.4 Z9-14:Ald	X	X	X							
9.8 16:Ald	X	X	X							
1.6 Z9-16:Ald	X	X	X							
132.4 Z11-16:Ald	X	X	X							
1.2 Z9-14:OH	X	X	X							
49.8 Z11-16:OH	X									
6.2 Z11-16:OH			X							
	Total male capture									
1984 (6 nights, 4 replicates)	10ab	0b	35a	22ab						
Rubber septa										
66.5 14:Ald					X					
58.7 Z9-14:Ald					X	X	X	X		X
264.0 16:Ald					X					
39.1 Z9-16:Ald					X			X		
3,319.5 Z11-16:Ald					X	X	X	X	X	X
31.3 Z9-14:OH					X		X	X		
152.5 Z11-16:OH					X	X	X	X	X	
	Total male capture									
1985 (14 nights, 5 replicates)					61a	79a	67a	67a	1b	—
1986 (18 nights, 6 replicates)					18b	36a	—	—	0b	0b
1987 (14 nights, 3 replicates)					28a	31a	—	—	0b	0b

^aTreatment 0 = virgin female iris borer. The Xs in each column indicate composition of each treatment. Male capture values in each row followed by the same letter are not significantly different from one another according to Duncan's multiple range test ($\alpha = 0.05$).

trapping test (table 2) indicated that this assumption was sound because the 96:4 ratio caused capture of more males than the 73:27 ratio or the virgin females. Thus, the 96:4 Ald-OH ratio probably is a better approximation of the ratio produced by the female than is the 73:27 ratio found in the ovipositor extracts. Similar results were obtained by Raina et al. (1986) in a study of the female sex pheromone of another noctuid moth, *H. phloxiphaga*. They found that a combination of Z11-16:Ald: Z11-16:OH was critical for elicitation of optimal response from males and that proportionately less alcohol than that

found in female extracts was the more effective male lure. Such a phenomenon has also shown to be the case for *H. virescens* (Ramaswamy et al., 1985; Shaver et al., 1987); with levels of alcohol at 0.25–1% of Z11–16:Ald field trap captures were enhanced, but at 5.9% Z11–16:OH (ca. the highest amount found in gland extracts according to Klun et al., 1980) captures were suppressed. In case of the iris borer, the question of what ratio is actually released by the female can only be answered definitively by analysis of the volatiles produced by the females. The 1984 trapping test definitively showed, however, that Z11–16:OH was an essential pheromonal component; no males were captured when it was deleted from the stimulus.

Data collected in field tests from 1985 through 1987 (Table 2) revealed that the minimum set of compounds required to effectively cause capture of iris borer males was a mixture of Z11–16:Ald, Z11–16:OH, and Z9–14:Ald (94:4:2). This mixture can be used as a male attractant for monitoring flights of the pest.

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CHEMICAL INHIBITION OF FIRE-PRONE GRASSES BY FIRE-SENSITIVE SHRUB, *Conradina canescens*

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Abstract—In an investigation of potential chemical activity of fire-sensitive shrubs in Florida's sand pine scrub community, bioassays of foliar washes of *Conradina canescens* showed significant inhibitory activity on three native grasses that are known to fuel frequent surface fires; inhibition was concentrated seasonally in spring and summer. Application of runoff from *Conradina* leaves to one of the grasses caused a 50% reduction in growth over a 20-week period. Isolation of the biologically active fractions from the fresh leaves of *C. canescens* yielded numerous monoterpenes, a number of which were identified from a GC-MS reference library and/or MS comparison to authentic compounds: 11 from the diethyl ether extract, 11 from steam distillation, and four from the foliar leaf wash. Numerous other monoterpenes present in the extractions were unknown. The terpenoid fraction completely inhibited seed germination of one of the native grasses and of lettuce. Saturated aqueous solutions of nine of the monoterpenes inhibited germination and radicle growth of two native grasses. SEM views of the leaf surfaces of *Conradina* reveal secretory trichomes that appear to be the source of the monoterpenes as well as the triterpene, ursolic acid. The biological activity of *C. canescens* as a fire-sensitive component of the scrub community is reviewed in light of the chemical evidence.

Key Words—Allelopathy, fire, monoterpenes, trichomes, *Conradina canescens*, *Pinus*, *Schizachyrium scoparium*.

INTRODUCTION

Two different plant communities exist on the upland, well-drained sands of Florida: the sand pine scrub characterized by sand pine [*Pinus clausa* (Chapm. ex Engelm.) Vasey ex Sarg.] with a dense shrub cover but no herbaceous ground cover, and the sandhill or high pineland dominated by longleaf pine (*P. palustris* Mill.) with a complete graminoid ground cover but few shrubs (Chapman, 1932; Laessle, 1958; Garren, 1943). Fire is a frequent feature of the sandhill, burning through the deciduous surface fuels every three to eight years (Williamson and Black, 1981). However, healthy scrub, which is evergreen, burns infrequently, about once in 50 years or once per generation of the dominant sand pine, which regenerates via serotinous cones (Harper, 1915; Richardson, 1977). As scrub and sandhill communities both occur on upland sandy soils, they are intermingled and contiguous throughout the Gulf Coastal Plain. The difference in fire susceptibility is remarkable in that fires sweep through the sandhills until they encounter patches or strands of scrub, where often they are extinguished by changes in the fuel and live vegetation. Webber (1933) called the scrub "a fire-fighting machine," although recognition of the differences between scrub and sandhill trace back to the last century. Nash (1895) noted that "The scrub flora is entirely different from that of the high pine land, hardly a single plant being common to both; in fact these two floras are natural enemies and appear to be constantly fighting each other. . . .," and Whitney (1898) reiterated that "it is an impressive sight to stand at the border line between the scrub and the high pine land and notice the difference in the character of the vegetation."

In the absence of surface fires in the sandhills, the woody species from the scrub will colonize (Laessle, 1958; Veno, 1976). They grow well in the sandhills, often faster than in the scrub, unless a surface fire sweeps through the graminoid ground cover and kills them (Veno, 1976; Hebb, 1982). Large shrubs and especially groups of shrubs may escape mortality, if graminoid growth around them has been suppressed. Therefore, we have been investigating the hypothesis that shrubs from the scrub produce chemicals that inhibit the growth of the grasses and perhaps pines, which provide the fuel for surface fires that otherwise would kill the shrubs. In particular, we are focusing on the early colonizers of the scrub community.

One such species is *Conradina canescens* (Torr. & Gray) (Lamiaceae), a small, evergreen mint, that is endemic to the sand pine scrub community. Its distribution is highly disjunct with two major populations—one on the Gulf coast from Horn Island, Mississippi, to just south of Tallahassee, Florida, and the other concentrated on Florida's Central Ridge in Highlands and Polk counties. In both locations the species is a relatively common shrub, reaching 2 m in height where scrub vegetation is colonizing and in full sunlight, but it diminishes in abundance under the canopy of sand pine and the subcanopy of oaks,

characteristic of mature scrub. The small (2×10 mm), recurved leaves of *Conradina* release a strong mintlike or terpenoid odor when crushed, whereas undamaged leaves emit no odor.

METHODS AND MATERIALS

Foliage from several *Conradina* shrubs was collected monthly from a site near Sun Ray, Florida. Care was taken to cut the stems so as not to tear the leaves in order to prevent the release of internally bound compounds. In the laboratory, 50 g of foliage was placed in 500 ml of distilled water for 24 hr under refrigeration at 8°C. The resulting leaf wash was filtered, and 5 ml was added to 30 target seeds placed on a sheet of Whatman No. 1 filter paper in a 10-cm-diameter Petri dish (Richardson, 1985; Williamson et al., 1988). Seeds of three native sandhill grasses, *Schizachyrium scoparium* (Michx.) Nash, *Andropogon gyrans* Ashe, and *Leptochloa dubia* (HBK.) Nees, were used as target species, each in four replicate dishes kept in the dark at 26°C. Control dishes given distilled water were not adjusted for pH or osmolarity because the *Conradina* leachates were within ranges (5–7 pH, 2–9 mOsm) that did not influence seed germination and growth.

Results of the treatment means (T) were compared to the control means (C) each month in a one-way analysis of variance (ANOVA) with four samples per monthly treatment. The magnitude of inhibition or stimulation is reported as RI, a response index that gives the percentage inhibition of the treatment relative to the control (a negative RI) or the percentage inhibition of the control relative to the treatment (a positive RI, when stimulation occurs). RI values allowed parametric comparisons (Williamson and Richardson, 1988).

A second bioassay was performed by misting water (6 mm/hr) over potted *Conradina* shrubs in a growth chamber and collecting the foliar runoff in glass funnels. Then the runoff was used to water flats ($5 \times 25 \times 60$ cm) of sandhill soil sown with 300 seeds of *Andropogon gyrans*. Control flats were given equal amounts of water. Flats were fertilized to field capacity every 18 days with Peters 20:20:20 (N/P/K). Throughout the experiment the seedlings were thinned only when a plant's leaves reached beyond the stems of its neighbors, so thinning occurred when plants reached a common size, but not necessarily at the same time. After 140 days, each remaining plant was removed from the flat and oven-dried at 105°C for three days, and the weights of leaves and roots were recorded.

Plant material for chemical analysis was collected from Dauphin Island, Alabama, on October 8, 1983, and again from Perdido Key, Alabama, on June 15, 1984. The sites are within 50 km of each other and probably are part of the same population.

Crude extracts of air-dried stems and of air-dried leaves were obtained by sequential use of petroleum ether (PE), diethyl ether (EE), and ethyl alcohol (EtOH) as follows: plant material was soaked in the solvent for 48 hr at 8°C, then filtered by suction and dried before soaking in the next solvent (de la Peña, 1985). Thereafter, the plant material was washed with 60°C ethyl alcohol and finally EtOH-Soxhlet extracted. Solvents were rotary evaporated below 50°C.

Saturated aqueous solutions were prepared from each of the five residues of the crude extracts of both stems and leaves and then tested for biological activity with Petri dish bioassays on *Schizachyrium scoparium*, the same native grass employed in the monthly bioassays. Replication varied from two to four dishes of 30 seeds. In addition, parallel bioassays were performed with commercial lettuce (*Lactuca sativa*).

Inhibitory activity was concentrated predominantly in the EE fraction from the leaves, so further chemical analysis was concentrated there. Silica gel column chromatography of the EE fraction yielded 80 fractions, which were combined according to results of thin-layer chromatography (TLC) into 11 fractions for subsequent bioassays. Biological activity was again concentrated in one sample, EE 6-7, which caused nearly complete inhibition of seed germination. Fractionation of EE 6-7 on a silica gel column yielded 59 fractions, which were combined according to TLC results to give 19 fractions for bioassays; from these, one sample, EE 6.16-6.24 was as inhibitory as EE 6-7. The EE 6.16-6.24 was analyzed using gas chromatography-mass spectral analysis (GC-MS) to identify the components of the mixture. The GC-MS analyses were performed on a Hewlett-Packard model 5985 instrument. A 30-m bonded silica capillary column was used for the GC-MS analysis, under the following conditions: injection temperature 250°C, column temperature program 60° for 1 min., followed by 5°/min. increases to 210°, temperature retained at 210° for 5 min. Compounds were identified by using the computerized MS spectral search system from Hewlett-Packard: Flavor-Fragrance, 59817D National Bureau of Standards Subset Library. Identities of some compounds were further corroborated by comparison of retention times and mass spectra to known standards.

Because many of the compounds in the EE active fraction appeared to be volatile organics, a steam distillation of *Conradina* foliage was undertaken. Fresh leaves in distilled water were boiled with the vapors trapped in an attached condenser. The distillate was extracted with diethyl ether, dried with anhydrous magnesium sulfate, filtered, and concentrated in a rotatory evaporator. The residue, labeled SD (steam distillation), was analyzed (GC-MS) for identification of compounds.

In order to determine compounds that might leach from foliage in precipitation, fresh leaves of *Conradina* were extracted with distilled water as follows: 360 g were twice soaked in 3 liters of distilled water for 3 days at 8°C, and the aqueous extract reextracted with ethyl acetate; the organic layer was dried with

anhydrous magnesium sulfate, filtered, and the solvent rotary evaporated. The 0.6-g residue, labeled Soak, was analyzed (GC-MS) to identify components of the mixture.

Nine of the monoterpenes, identified from the active fractions of *Conradina*, were tested for biological activity on two native grasses, *Leptochloa* and *Schizachyrium*, in triplicate Petri dish bioassays, as described earlier, 30 seeds per dish. Most of these monoterpenes are low-molecular-weight, nonpolar entities, whose solubilities in water have never been determined, so saturated aqueous solutions were prepared for the bioassays here.

Scanning electron micrographs of the surface of the leaves were examined for possible trichomes, as sources of production of active compounds. Both fresh leaves and fresh leaves dipped in an organic solvent, dichloromethane (DCM), were examined.

RESULTS AND DISCUSSION

The monthly bioassays of *Conradina* leaf washes showed similar effects on the three sandhill grasses, with *Leptochloa* experiencing the most inhibition, *Andropogon* the least, and *Schizachyrium* intermediate (Table 1). Simple ANOVAs of each month's data produced significant inhibition of germination in 52% of the monthly tests—in 88% for *Leptochloa*, 44% for *Schizachyrium*, and 25% for *Andropogon*. Inhibition of radicle length was about half as frequent—in 28% of the monthly tests overall, 50% for *Leptochloa*, 22% for *Schizachyrium*, and 12% for *Andropogon*. The frequency of the significant results suggested that inhibition occurred regularly, but not every month.

Species differences in the magnitude of the monthly responses followed

TABLE 1. GERMINATION AND RADICLE LENGTH RESPONSES OF THREE SANDHILL GRASSES TO LEAF WASHES OF *Conradina canescens* RELATIVE TO WATER CONTROLS

	<i>Leptochloa</i>	<i>Schizachyrium</i>	<i>Andropogon</i>	Total
Months tested	8	9	8	25
Significant inhibition				
Of germination	7	4	2	13
Of radicle length	4	2	1	7
Mean monthly response (RI)				
Of germination	-0.34 ± 0.17 ^a	-0.17 ± 0.37	-0.09 ± 0.17	-0.22 ± 0.28 ^a
Of radicle length	-0.13 ± 0.14 ^b	-0.10 ± 0.22	+0.03 ± 0.39	-0.08 ± 0.25 ^b

^aTreatment was significantly different from the control at $P \leq 0.01$.

^bTreatment was significantly different from the control at $P \leq 0.05$.

closely their differences in the frequency of significant inhibitions. Average percent inhibition of germination was 22% relative to the control overall—34% for *Leptochloa*, 17% for *Schizachyrium*, and 9% for *Andropogon* (Table 1). Inhibition of radicle length was only 8% of the control overall—13% for *Leptochloa* and 10% for *Schizachyrium*, whereas the control was 3% less than the treatment (stimulation) with *Andropogon* (Table 1). For the three species combined, monthly inhibition averaged 22% for germination and 8% for radicle length.

Monthly variation in bioassay effects was large but by no means random. Inhibition of germination of the three grasses rose to a sharp peak in June and was distinctly absent in the winter months (Figure 1). Inhibition of radicle length seemed to be concentrated in the spring, with less predictable seasonal variation the rest of the year. The monthly germination responses (RI) were negatively correlated with 30-year averages for precipitation ($r = -0.74$, $P = 0.01$) and temperature ($r = -0.72$, $P = 0.01$), so inhibition of germination was concentrated in the warm, wet months—exactly the growing season in central Florida (Figure 1). Radicle length responses were not correlated with precipitation ($r = -0.14$, $P = 0.70$) or temperature ($r = -0.49$, $P = 0.15$).

Application of leaf mist collected from *Conradina* to seedlings of *Andropogon gyrans*, growing in flats of sandhill sand, caused significant ($P = 0.05$)

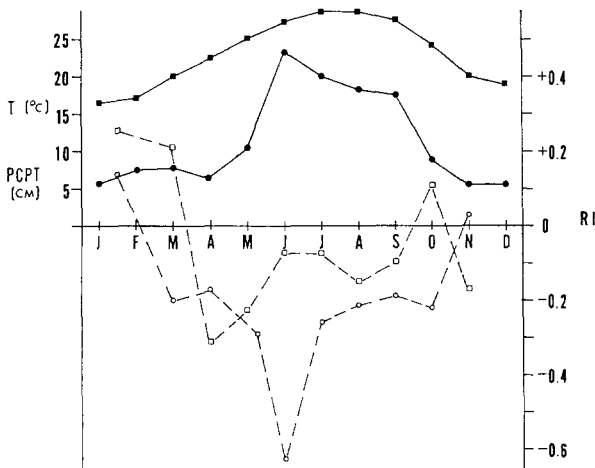


FIG. 1. Dashed lines show the mean monthly responses (RI) of three sandhill grasses treated with leaf washes of *Conradina canescens* relative to grasses given water controls. Negative RI values are inhibition and positive RI values are stimulation for germination (circles) and radicle length (squares). Solid lines show the 30-year monthly precipitation mean (circles) and the 30-year monthly temperature mean (boxes) near the site (NOAA 1983).

inhibition of growth at the end of the 140-day experiment. Seedlings watered with *Conradina* mist weighed only 48% of the control plants (2.4 ± 0.2 g versus 4.0 ± 0.6 g). Root weights from the *Conradina* treatment were not different from the control (1.0 ± 0.3 g versus 0.9 ± 0.1 g), but leaf weights in the treatment were only 43% of the control (1.4 ± 0.3 g versus 3.1 ± 0.5 g). Height of the grasses given the treatment was only half that of the control plants (43 ± 4 cm versus 86 ± 7 cm). Thus, inhibition of growth was quite strong in the mist test but was not apparent in the Petri dish bioassays with *Andropogon*.

Two possible mechanisms could account for the difference. Growth in the Petri dish tests was limited to one week, but in the mist experiment growth was continued for 20 weeks; therefore, inhibition of *Andropogon* may be limited to later in development. Second, in the Petri dish bioassays, the *Conradina* leachate was applied only once at the beginning of the bioassay, which requires seven days for germination followed by seven days for growth, but in the mist experiment the leaf rinse was applied every three days. If the active compounds are volatiles, as is suggested below, then they may have dissipated from the Petri dishes before radicle growth commenced. The dishes were not sealed, nor were any of the bioassays performed in limited gas volumes, as has been the case in other bioassay studies of volatiles (Muller et al., 1964; Muller, 1965; Muller and del Moral, 1966; Fischer, 1986).

Chemical analysis of *Conradina* foliage revealed a variety of monoterpenes, as is the case with other allelopathic mints (Muller et al., 1964; Muller, 1965; Muller and del Moral, 1966; Fischer, 1986). Many of them were known compounds found in the GC-MS reference library and then confirmed by comparison to standards (Table 2). The diethyl ether (EE) extract produced 11 known monoterpenes, many of them, such as 1,8-cineole, camphor, borneol, and *p*-cymene, inhibitors of seed germination and plant growth (Fischer, 1986). The steam distillation produced 11 known monoterpenes, and the leaf soak produced four known monoterpenes (Table 2). Some (seven of 11) of the known monoterpenes from steam distillation and all of them from the leaf soak (four of four) were also present in the EE extract. The GC traces of the steam distillation and the leaf soak revealed a large number of suspected monoterpenes (lower-molecular-weight compounds) that were not known from the reference library—an indication that *Conradina canescens* contains a highly unique complement of volatile compounds.

Petri dish bioassays of nine of the monoterpenes, applied to *Leptochloa* and *Schizachyrium*, revealed complete inhibition of germination by five compounds and statistically significant inhibition of germination in each case with one exception—cineole did not inhibit *Leptochloa* (Table 2). Where germination did occur, there was subsequent evidence for inhibition of radical growth in most cases, although sample sizes were limited by the small numbers of

TABLE 2. MONOTERPENES IN ACTIVE DIETHYL ETHER FRACTION (EE), STEAM DISTILLATION EXTRACT (SD), AND LEAF SOAK (SOAK) OF *Conradina*, WITH MS CONFIDENCE LEVEL, MS CONFIRMATION TO AUTHENTIC COMPOUND AND PERCENT INHIBITION OF GERMINATION AND RADICLE LENGTHS OF *Leptochloa* AND *Schizachyrium* AS PERCENT OF DISTILLED WATER CONTROLS

Compound	EE	SD	Soak	MS	Confirmed	<i>Leptochloa</i>		<i>Schizachyrium</i>	
						Germ.	Rad.	Germ.	Rad.
1,8-Cineole	+	+	+	95	yes	100	97	20 ^a	26 ^a
Camphor	+	+	+	89	yes	12 ^a	34 ^a	16 ^a	61
Pinocarveol	+	+	-	96	no				
Borneol	+	-	+	91	yes	0 ^a		8 ^a	47 ^b
Pinocamphone	+	+	-	97	no				
Myrtenal	+	+	-	95	yes	2 ^a	5 ^a	8 ^a	13 ^a
α -Terpineol	+	+	+	98	yes	0 ^a		0 ^a	
Myrtenol	+	+	-	97	yes	0 ^a		0 ^a	
Carveol	+	-	-	95	yes	0 ^a		0 ^a	
Carvone	+	-	-	91	yes	0 ^a		0 ^a	
Citronellol	+	-	-	90	no	0 ^a		0 ^a	
<i>p</i> -Cymene	-	+	-	98	no				
Sabinene	-	+	-	94	no				
1,6-Dihyrosylvestone	-	+	-	94	no				
Caryophyllene	-	+	-	97	no				

^aTreatment was significantly different from the control at $P \leq 0.01$.

^bTreatment was significantly different from the control at $P \leq 0.05$.

germinating seeds (Table 2). Thus, saturated aqueous solutions of most of the monoterpenes tested provided strong phytotoxicity. In these tests the Petri dishes were not sealed, and volatilization of the monoterpenes was quite evident from their odors in the test room, especially the first few days of the experiment. Exactly how such compounds are transmitted to the soil solution in nature remains an enigma, although in chaparral volatiles appear to settle and adsorb on lipophilic clay particles and seed/seedling membranes (Muller and del Moral 1966).

Florida's sandy soils are considerably less lipophilic, but monoterpenes may reach the soil in water runoff. One line of our research suggests that monoterpenes may be associated with biological surfactants that reduce their volatility and increase their solubility in water (Fischer, 1986; Fischer et al., 1988). In the case of *Conradina*, a known triterpene, ursolic acid, composed 6% of the dry weight of the plant and 50% of the crude EE extract (de la Peña, 1985). In acridine dye tests, ursolic acid produced micelles (de la Peña, 1985; Williamson et al., 1988). Therefore, the bioassays with monoterpenes alone (Table

2) may underestimate their activities in nature if they form micelles with ursolic acid or other organic compounds.

Scanning electron micrographs of the leaf surfaces of *Conradina* showed numerous nonglandular, filamentous trichomes as well as fewer glandular, spherical ones. The latter were surrounded and covered by the former, and cross-sections through the latter revealed secretory cells under a fold of the cuticle that harbored the secretion (Figure 2). Similar glandular and nonglandular trichomes are found in some, but not all, mint species (Metcalf and Chalk, 1965). Dipping the leaves in a polar solvent such as diethyl ether or dichloromethane (DCM) dissolved the fold of cuticle and the underlying secretions, whereas the filamentous trichomes remained intact. The resulting DCM extract contained monoterpenes as well as ursolic acid.

In summary, the preliminary results presented here indicate that aqueous washes of foliage of *Conradina canescens* seasonally inhibit germination of

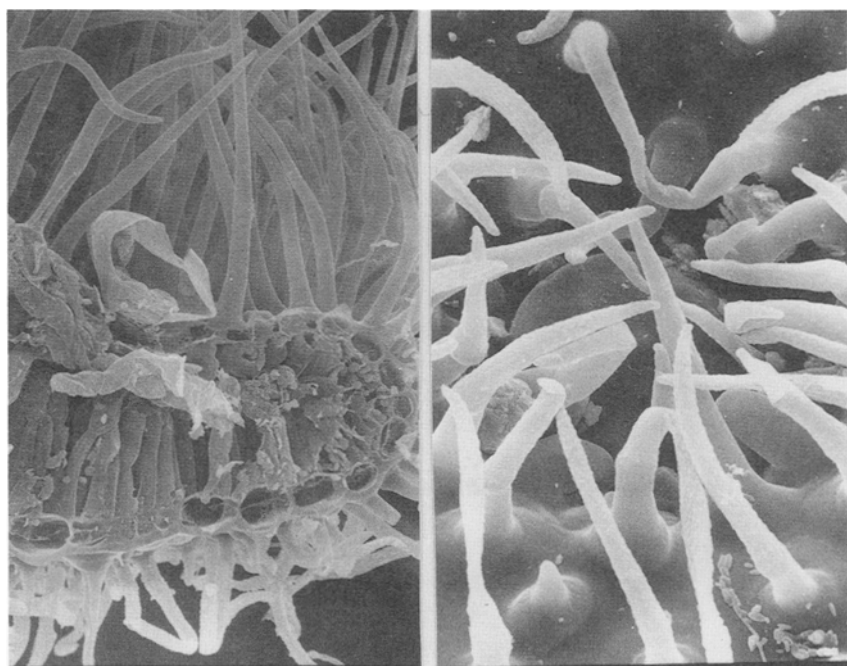


FIG. 2. At left is a cross-section through the leaf and through a glandular trichome on the leaf lower surface; trichome consists of the fold of cuticle, the pocket that contained the secretion, and the underlying secretory cells. At right is the upper leaf surface showing the nonglandular trichomes covering a spherical glandular trichome. Line in the lower right of each micrograph is 50 μm .

native grasses. The washes contain monoterpenes that are probably the source of the antibiosis. The monoterpenes are packaged on the leaf surface with ursolic acid as secretions of trichomes. The secretions are under the cuticle, but rinsing the leaves gives a leachate that contains the monoterpenes, so the leaves appear to provide a leachable source of phytotoxins. Thus, allelopathy may function to prevent the accumulation of fine fuel (grass litter) around *Conradina* shrubs, which otherwise suffer mortality in surface fires. The effects of the allelopathic inhibition relative to other kinds of biological interactions such as competition and herbivory have not yet been investigated, so its importance in nature is still unresolved.

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CHANGES IN LEAF MONO- AND SESQUITERPENE METABOLISM WITH NITRATE AVAILABILITY AND LEAF AGE IN *Heterotheca subaxillaris*

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Abstract—The concentration of leaf mono- and sesquiterpenes is greater in nitrate-limited than in nitrate-rich *Heterotheca subaxillaris* plants and is highest in young leaves and declines with leaf age. To determine whether rates of volatile terpene synthesis and/or loss vary with nitrate availability and leaf age, incorporation of ¹⁴C from photosynthetically fixed ¹⁴CO₂ and the subsequent loss of label was measured in plants grown under nitrate-limited and nitrate-rich conditions. ¹⁴C incorporation into mono- and sesquiterpenes was greater in nitrate-limited than in nitrate-rich plants and was highest in young leaves and declined with leaf age. Incorporation continued for several days after exposure, while loss of label was slow until leaves were 4–6 weeks old. These results suggest that the higher leaf volatile terpene content observed under nitrate limitation apparently results from increased synthesis per leaf and accumulation of mono- and sesquiterpenes in immature leaves of nitrate-poor plants. Furthermore, volatile terpene synthesis is highest in young leaves, declines with leaf age, and is very low in older leaves. Carbon used for synthesis of this pool may be derived from both current photosynthesis as well as carbon transported to young leaves from older leaves. These data are consistent with hypotheses that predict that greater levels of carbon-based chemical defenses occur in plants under nutrient limitation. The apparent low metabolic cost of maintenance (i.e., slow turnover) of the accumulated terpenoid pool would limit the energetic cost of volatile terpenes as a chemical defense.

Key Words—*Heterotheca subaxillaris*, Asteraceae, monoterpene, sesquiterpene, nitrogen, leaf age, metabolism, turnover, chemical defense, biosynthesis.

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INTRODUCTION

Reduced plant nitrate availability results in increased accumulation of leaf mono- and sesquiterpenes in *Heterotheca subaxillaris* and may reduce leaf consumption by herbivores (Mihaliak and Lincoln, 1985; Mihaliak et al., 1987). The higher leaf volatile terpene content with nitrate limitation could be due to increased synthesis resulting from changes in allocation of carbon and energy to the sites of terpene synthesis (i.e., in the secretory cells of glandular trichomes). Accumulation of volatile leaf terpenes is thought to be linked to the relationship between photosynthetic rate and the utilization of photosynthate within a leaf (Croteau et al., 1972b; Loomis and Croteau, 1980). Increased leaf nonstructural carbohydrate content in plants growing under low nitrogen conditions (Chapin, 1980; Chapin et al., 1986) may provide an enhanced carbon and energy source for terpene synthesis. Thus, assuming the rate of terpene catabolism is not affected by nitrate availability, increased terpene synthesis at low nitrate supply would result in a higher concentration of leaf terpenes. Alternatively, monoterpenes undergo turnover (Burbott and Loomis, 1969), and slower catabolism could also result in the accumulation of leaf volatiles in nitrate-poor plants.

The concentration of leaf mono- and sesquiterpenes is often highest in young leaves and declines with leaf age (Burbott and Loomis, 1969; Crankshaw and Langenheim, 1981; Mihaliak and Lincoln, 1985). Rapid synthesis of mono- and sesquiterpenes occurs in young leaf tissue (Burbott and Loomis, 1969; Croteau and Loomis, 1972). As leaves age, the rate of terpene synthesis is thought to decline, and the leaf volatiles that accumulated in long-term pools (i.e., in glandular trichomes) may be catabolized (Croteau et al., 1981; Croteau 1984).

The objective of the current experiment was to measure ^{14}C incorporation and loss from the leaf mono- and sesquiterpenes of *Heterotheca subaxillaris* to determine whether: (1) volatile terpene biosynthesis in young leaves of nitrate-limited plants is greater than in comparable leaves of nitrate-rich plants; (2) turnover of volatile terpenes differs with nitrate availability; and (3) volatile terpene synthesis declines with leaf age.

METHODS AND MATERIALS

Heterotheca subaxillaris (Lam.) Britton and Rusby (Asteraceae) (camphorweed) is a herbaceous annual/biennial species that occurs in old fields, disturbed sites, and open sandy areas throughout the southern United States. Forty-one mono- and sesquiterpenes have been identified from the leaves of *H. subaxillaris* (Lincoln and Lawrence, 1984). Seeds collected from a single maternal parent (to reduce genetic variation) at the Clemson University Sandhill

Agriculture Experimental Station near Columbia, South Carolina, were germinated on filter paper and then transferred to a nonnutritive potting media of perlite, vermiculite, and sand (1 : 1 : 1) when the plants were less than 1 week old. The plants were watered on alternate days with either tap water or one of two nutrient solutions, which differed only in the concentration of nitrate (either 1.5 mM or 15.0 mM nitrate) (Mihaliak and Lincoln, 1985) and were maintained on a 12-hr photoperiod in an environmental growth chamber (26°C day, 20°C night) under a light intensity of 260 $\mu\text{mol}/\text{m}^2/\text{sec}$ of photosynthetically active radiation.

Four rosette-stage plants per day were simultaneously exposed to $^{14}\text{CO}_2$ in a sealed Plexiglas chamber (26.63 liter volume). The labeling procedure began 1 hr after the start of the light cycle for all plants when $^{14}\text{CO}_2$ was generated from aqueous $^{14}\text{C}[\text{NaHCO}_3]$ (56 mCi/mmol, 25 μCi per plant) by injecting perchloric acid through a rubber septum into a beaker containing the $^{14}\text{C}[\text{NaHCO}_3]$. During exposure, air in the chamber was circulated with a small electric fan and light was provided using a 400 W mixed-metal halide high-pressure sodium lamp (650 $\mu\text{mol}/\text{m}^2/\text{sec}$ of photosynthetically active radiation at plant height). Air temperature in the chamber remained within 2°C of the ambient laboratory temperature (24°C) during the exposure period. After a 30-min exposure, unincorporated $^{14}\text{CO}_2$ was flushed from the Plexiglas chamber through KOH traps and the plants were returned to the environmental chamber.

Leaves of four different ages from rosettes in each nitrate treatment were analyzed: 1 week old (immature, expanding leaves), 2 weeks old (at or near full expansion), 4 weeks old (mature leaf), and 6 weeks old (senescent necrosis beginning). Each observation reported for leaves 1 or 2 weeks old when labeled is an average of 2 leaves sampled from different plants. The observations reported for 4- and 6-week-old leaves are usually for a single leaf sample. After removal, leaves were immediately placed on dry ice, transported to the laboratory, and weighed. The leaves were then either prepared for analysis or briefly stored at -70°C until sample preparation occurred.

The volatile terpene fraction of each leaf was captured using a microdistillation apparatus modeled after that of Godefroot et al. (1981) and Rijks et al. (1983). The apparatus is designed to collect the steam-distillable, pentane-soluble fraction from small amounts of leaf material and allows for efficient capture (routinely greater than 90%) of volatile mono- and sesquiterpenes from a single leaf. The leaf was boiled in a 50-ml round-bottom flask that contained 10 ml of distilled water and a known amount of internal standard (*n*-tetradecane). Approximately 3 ml of pentane was used to trap the volatiles in a 10-ml round-bottom flask, and Teflon boiling chips were added to both flasks. To ensure that pentane extraction would begin before any volatile material was distilled from the leaf, the flask containing pentane was heated for approximately 3 min before heating the flask containing the leaf and water. After a 1-hr distillation,

the pentane (which contained the volatiles) was recovered and evaporated under a stream of nitrogen to a volume of approximately 75 μ l.

The volatile terpene content of each leaf was determined by gas chromatography using a SP-1000 fused silica capillary column, flame ionization detector, and a digital integrator (GC temperature program of 50–175°C at 5°C/min. with 5- and 18-min initial and final isothermal periods, respectively). After GC analysis, ^{14}C incorporation into the volatile terpenes was quantitated using a liquid scintillation counter (Packard Tricarb Series 4000; Beckman Ready-Solv NA was used as the scintillation cocktail). No quenching occurred when excess amounts of distillate from unlabeled *H. subaxillaris* leaves was added to a labeled sample. The presence of the measured ^{14}C in the mono- and sesquiterpenes was confirmed by gas radiochromatography using a gas chromatograph equipped with a thermal conductivity conductor, an AT-1000 stainless-steel column (150°C isothermal analysis) and a Packard model 894 Gas Proportional Counter.

RESULTS

Incorporation of ^{14}C into mono- and sesquiterpenes was greater in immature leaves of nitrate-limited plants than in nitrate-rich plants. Under nitrate-rich conditions, ^{14}C incorporation per leaf into the volatile terpene pool of immature leaves showed a pattern of rapid incorporation and loss over the first 4 hr followed by another period of increased incorporation beginning 10 hr after exposure (Figure 1). Immature leaves of nitrate-limited plants did not exhibit, within the first 6 hr, the same pattern as was observed under nitrate-rich conditions. Instead, a steady increase in total radioactivity per leaf of the volatiles occurred over the first 24 hr following exposure (Figure 1). By 24 hr after exposure, incorporation into the volatile terpene fraction of immature leaves of nitrate-limited plants was nearly twice as high as in comparable leaves of nitrate-rich plants on a per leaf basis, and nearly fivefold higher on a per milligram leaf basis. There was no apparent diurnal trend in the quantity of leaf volatile terpenes over this period (Figure 1, inset).

As leaves aged, incorporation per leaf into the volatiles declined in both treatments. The 2-week-old leaves of nitrate-limited plants exhibited a rapid increase and loss of label similar to immature leaves of nitrate-rich plants, including a second increase in specific activity 10 hr after the exposure (Figure 1). Under nitrite-rich conditions, little initial incorporation occurred during the first 6 hr after exposure in 2-week-old leaves, but the level of incorporation had risen by 24 hr, although to a lower specific activity than in immature leaves. The 4- and 6-week-old leaves of plants under both nitrate regimes exhibited essentially no incorporation into the volatile terpenes (Figure 1).

The highest levels of ^{14}C incorporation occurred between 1 and 28 days

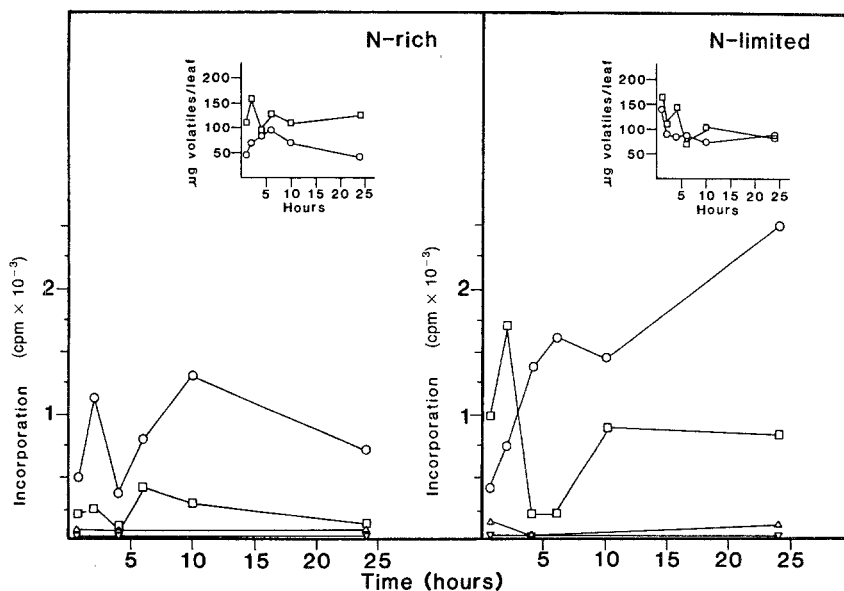


FIG. 1. Short-term time course of ^{14}C incorporation from $^{14}\text{CO}_2$ into volatile terpenes in leaves of four ages from *Heterothecha subaxillaris* rosettes grown under nitrate-limiting and nitrate-rich conditions. Inset: The volatile terpene content of the 7- and 14-day-old leaves. \circ = 1 week old, \square = 2 weeks old \triangle = 4 weeks old, ∇ = 6 weeks old.

after labeling in the expanding leaves of plants from both treatments (Figure 2). Incorporation per leaf into the terpene pool was, on average, 2.1 times higher in expanding leaves (1 week old) from nitrate-limited plants than in equivalent leaves from nitrate-rich plants. Over the course of the experiment, the variation in incorporated ^{14}C in the mono- and sesquiterpenes between the duplicate samples for each measurement was approximately 40% of the mean. In both treatments, incorporation into 2-week-old leaves was lower than in immature leaves, and the 4- and 6-week-old leaves of plants exhibited essentially no incorporation during the entire experiment (Figure 2).

Loss of label from the terpene pool occurred between 28 and 42 days after exposure in both treatments. By the 42-day sampling period, senescence had begun in the leaves of plants from both nitrate treatments. Similarly, loss of label from 2-week-old leaves of nitrate-rich plants occurred between 10 and 28 days after exposure. No trend in loss of label from the terpene pool was observed among leaves from 2-week-old, nitrate-limited plants.

The volatile terpene concentration was significantly higher in leaves of nitrate-limited plants (which show greater levels of incorporation into the volatile terpenes) than of nitrate-rich plants ($F = 7.4$, $P < 0.01$, $df = 1, 57$, two-

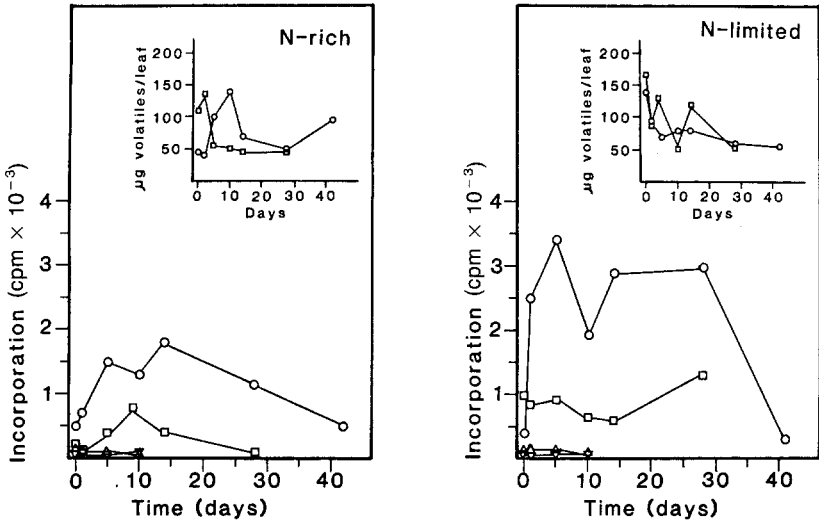


FIG. 2. Long-term time course of ^{14}C incorporation from $^{14}\text{CO}_2$ into volatile terpenes in leaves of four ages on *Heterotheca subaxillaris* rosettes growth under nitrate-limiting and nitrate-rich conditions. Inset: The volatile terpene content of the 7- and 14-day-old leaves. \circ = 1 week old, \square = 2 weeks old, \triangle = 4 weeks old, ∇ = 6 weeks old.

way ANOVA), and the quantity of volatile terpenes declined with leaf age in both treatments ($F = 7.7, P < 0.001, df = 3,55$, two-way ANOVA; interaction not significant, $P = 0.6$) (Figure 3A). The decline in leaf volatile terpene content as leaves aged coincided with the loss of label from older leaves that

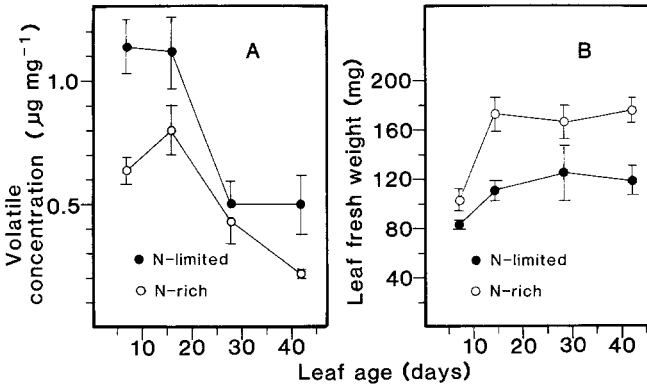


FIG. 3. (A) The mean volatile terpene concentration (\pm SE) of four ages of leaves from *Heterotheca subaxillaris* rosettes grown under nitrate-limited and nitrate-rich conditions. (B) The mean fresh weight (\pm SE) of *H. subaxillaris* leaves from plants grown under nitrate-limited and nitrate-rich conditions.

had been labeled when immature (Figure 2). The leaves of nitrogen-rich plants were fully expanded after 14 days and leaves of nitrate-limited plants were fully expanded between 14 and 28 days (Figure 3B). Thus, ^{14}C incorporation into the volatile terpenes occurred primarily in expanding leaves.

The enhanced terpene synthesis in immature leaves of nitrate-limited plants is also observed when differences in synthesis are compared on a leaf weight basis. The mass of 1-week-old leaves from nitrate-limited plants is 20% less than comparably aged leaves from N-rich plants, whereas the rate of terpene synthesis and the mass of volatile terpenes per leaf are both greater in these smaller leaves (Figure 1). Similarly, changes in volatile concentration over leaf life are influenced by changes in leaf weight (Figure 3), but appear to result more from differences in synthesis early in leaf growth.

DISCUSSION

The higher concentration of leaf mono- and sesquiterpenes with decreased nitrate availability apparently results from increased synthesis into a slowly catabolized (metabolically stable) volatile terpenoid pool in young leaves. The enhanced biosynthesis may have resulted from greater carbon availability at the site of synthesis. Mono- and sesquiterpene synthesis is thought to be an energy deficient process because the isolation of volatile terpene metabolism (i.e., in secretory cells of glandular hairs) restricts the availability of carbon and energy to the sites of terpene synthesis (Croteau et al., 1972b, Loomis and Croteau, 1980). However, leaf nonstructural carbohydrate content increases with nitrate limitation (Fritsch and Jung, 1984; McNaughton, 1985; Chapin, 1980; Chapin et al., 1986). Metabolism of this carbohydrate pool could enhance carbon and energy availability for terpenoid synthesis by increasing the local supply of acetyl-CoA and energy to terpenoid-producing cells (Croteau et al., 1972b). On the other hand, increasing the carbon supply to peppermint plants via an enriched CO_2 atmosphere does not alter volatile terpene accumulation or presumably the local availability of carbon for synthesis (Lincoln and Couvet, 1989). Thus, changes in the quantity of leaf volatiles that have been observed in response to a range of abiotic environmental factors (e.g., Lincoln and Langenheim, 1979; Mihaliak and Lincoln, 1985; Gershenzon, 1984) may result from altered rates of volatile synthesis.

Photosynthate used for volatile terpene biosynthesis may be derived from current photosynthate as well as from carbon transported to young leaves from older leaves. The rapid ^{14}C incorporation into and loss from leaf volatiles of plants from both nitrate treatments (i.e., over the first 6 hr after exposure) suggests that current photosynthate is utilized for volatile terpenoid biosynthesis. Studies on other classes of terpenoids also suggest that current photosynthate may be used in synthesis of isoprenoids (Keene and Wagner 1985; Ramachan-

dra Reddy and Rama Das, 1987; Schulze-Siebert and Schultz, 1987). Incorporation of $^{14}\text{CO}_2$ into a metabolically stable volatile terpenoid pool was apparent 6–10 hr after exposure and continued for several days, suggesting that carbon could be transported into the immature, terpene-synthesizing leaves from older leaves. Furthermore, we were able to measure ^{14}C in the volatiles of immature leaves from plants that had been exposed to $^{14}\text{CO}_2$ 42 days prior to harvesting these leaves, albeit at low levels (up to 230 cpm ^{14}C above background). Incorporation into the volatiles of these immature leaves must have come from a carbon pool assimilated weeks before the sampled leaves were initiated.

The rate of volatile terpene biosynthesis is greatest in young leaves and declines precipitously with leaf age. Furthermore, the terpene pool that accumulated when the leaf was expanding is lost through catabolism, transport, or volatilization only when the leaf nears senescence. The long-term volatile terpene pool is thought to be secreted into glandular trichomes (Croteau, 1984) and, once secreted, the volatiles are apparently not susceptible to turnover or loss until the leaf nears senescence. Thus, the decreased concentration of mono- and sesquiterpenes commonly observed as leaves age (Figure 3) (Burbott and Loomis, 1969; Crankshaw and Langenheim, 1891; Mihaliak and Lincoln, 1985) apparently results initially from a reduced rate of volatile terpene synthesis as leaves become fully expanded, but ultimately from loss of the metabolically stable terpene pool.

Other detailed studies that have measured short-term $^{14}\text{CO}_2$ uptake and loss from mono- and sesquiterpenes of young leaves indicated that turnover occurred rapidly (i.e., less than 24 hr) with the highest level of incorporation occurring approximately 6 hr after labeling (Burbott and Loomis, 1969; Croteau et al., 1972a). The current results show two patterns: an initial uptake and loss of label and, additionally, maximum incorporation several days after exposure and loss several weeks later. The contrast between the results of the current study and some previous investigations may be due to previous studies being conducted with plant cuttings (Burbott and Loomis, 1969; Croteau et al., 1972a), while the current experiment was conducted with intact plants. An early study of $^{14}\text{CO}_2$ incorporation into leaf volatile terpenes of intact plants (*Pinus silvestris*) also demonstrated maximum incorporation into the leaf volatiles several days after exposure (Sukhov, 1958).

Mono- and sesquiterpenes are thought to function as chemical defenses against leaf-eating insects because a higher leaf volatile terpene content often reduces leaf consumption by herbivores (e.g., Mabry and Gill, 1979; Harborne, 1982; Mihaliak et al., 1987). The increased synthesis of leaf volatiles under nitrate limitation in *H. subaxillaris* is consistent with hypotheses that suggest that plants growing in nitrogen-limiting environments will allocate more carbon to leaf chemical defense than plants in nitrogen-rich environments (Janzen,

1974; Bryant et al., 1983; Coley et al., 1985; Mihaliak and Lincoln, 1985). Furthermore, the current results are consistent with the suggestion that carbon allocation to allelochemicals should reflect leaf value (i.e., the potential net carbon gain from the leaf tissue) (Rhoades, 1979; Gulmon and Mooney, 1986). Carbon allocated to synthesis of volatile terpenes is highest in young leaves, declines with leaf age, and does not occur in old leaves. Loss (e.g., catabolism) from the volatile terpene pool of older, less productive leaves suggests a reduced investment in defense as well as a possible recovery of carbon and energy that could be used for growth, reproduction, or the chemical defense of new leaves.

Production of plant chemical defenses that exhibit rapid turnover are thought to be energetically "costly" for plants to produce and maintain (Coley et al., 1985; Gulmon and Mooney, 1986). In the current experiment, the major pool of leaf volatiles was a metabolically stable pool in which turnover appears to be slow until leaves near senescence. This lack of rapid turnover would limit the energetic cost of mono- and sesquiterpenes as leaf chemical defenses.

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RESPONSES BY CANIDS TO SCENT GLAND SECRETIONS OF THE WESTERN DIAMONDBACK RATTLESNAKE (*Crotalus atrox*)

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Abstract—Many snakes discharge malodorous fluids from paired scent glands in the base of the tail when they are disturbed. A number of authors suggest that these secretions repel predators. Scent gland secretions of the western diamondback rattlesnake (*Crotalus atrox*), or dichloromethane extracts of them, were presented to coyotes (*Canis latrans*) in three field tests, and to domestic dogs (*Canis familiaris*) in two kennel tests, to determine whether responses of possible benefit to snakes are elicited. Free-ranging coyotes visited and rubbed and rolled at stations containing scent gland secretions in perforated plastic capsules more frequently than at those containing untreated or dichloromethane-treated capsules. Responses to dichloromethane extracts of scent gland secretions subjected to rotary evaporation were not significantly different from those to dichloromethane. Pure and mixed breeds of dogs presented with filter papers treated with dichloromethane or a dichloromethane extract of scent gland secretions mouthed (licked, bit, or ate) secretion-treated papers more frequently. Staffordshire terriers presented with filter papers treated with dichloromethane or dichloromethane extracts of snake scent gland and alligator (*Alligator mississippiensis*) paracloacal gland secretions exhibited urination postures to snake secretion-treated papers more frequently than to dichloromethane-treated papers, but responses to snake- and alligator-treated papers did not differ significantly. There was no indication that canids are repelled by scent gland secretions.

Key Words—Canidae, coyote, *Canis latrans*, dog, *Canis familiaris*, rattlesnake, *Crotalus atrox*, scent glands.

INTRODUCTION

Reptiles produce a variety of secretions thought to repel predators. In only a few studies, however, have their natural products been tested for antipredator activity (Eisner et al., 1977; Kool, 1981; Weldon, 1988).

Scent glands are paired exocrine organs in the tail of all snakes (Bellairs, 1950; Whiting, 1969). These glands typically release fluids, which are thought to discourage predators (Fitch, 1960, 1965; Burkett, 1966; Klauber, 1972), from two openings at the posteriolateral margin of the cloaca when snakes are disturbed. Several species of ants (Gehlbach et al., 1968) and several snakes (Watkins et al., 1969), including ophiophagous species, are repelled by the scent gland secretions of the Texas blindsnake (*Leptotyphlops dulcis*). A preliminary test indicated that some North American carnivores are reluctant to approach or eat food treated with desert kingsnake (*Lampropeltis getulus splendida*) secretions (Price and LaPointe, 1981), but there are no quantitative tests of the reactions by mammalian predators to these substances.

Rattlesnakes (genus *Crotalus*) typically discharge, and sometimes spray, scent gland fluids when molested (Klauber, 1972; Duvall et al., 1985). Since many mammals are resistant to snake venom (Perez et al., 1978), defenses other than striking and envenomation—such as scent gland discharge—may be useful against these predators. Canids are one group of carnivores with which rattlesnakes interact. We report here tests of the reactions of coyotes (*Canis latrans*) and domestic dogs (*Canis familiaris*) to scent gland secretions of the western diamondback rattlesnake (*Crotalus atrox*).

This study was conducted to determine whether responses of possible benefit to snakes are elicited in canids by scent gland secretions. Our field tests were designed primarily to test coyotes, although data on other vertebrates visiting our scent stations are presented. The series of field experiments involved different methods of chemical sample preparation or presentation, since the results of each test suggested questions that were addressed by modifications in subsequent designs. None of the field tests involved direct observation of the behavior of coyotes. Rather, inferences were made regarding coyotes' responses on the basis of impressions left in the soil or from other telltale deposits. Domestic dogs were tested to allow direct observation of canids' responses to scent gland secretions.

METHODS AND MATERIALS

Coyotes

Responses to rattlesnake scent gland secretions were conducted at the Chaparral Wildlife Management Area, a 6151-hectare ranch located in the Rio Grande Plains region of Texas in Dimmit and La Salle counties, and at the La

Copita Research Area, a 1093-hectare ranch located in Jim Wells County, approximately 15 km south of Alice, Texas. The average annual rainfall at the Chaparral and La Copita areas is 84 and 68 cm, respectively. The vegetation of both areas is dominated by honey mesquite (*Prosopis glandulosa*) and includes brazil (*Condalia obovata*) and prickly pear cactus (*Opuntia rigidula*). In addition to coyotes, eastern cottontail rabbits (*Sylvilagus floridanus*), striped skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), badgers (*Taxidea taxus*), javelina (*Tayassu tajacu*), and rodents were observed at both sites at the time of testing. Five *C. atrox* were seen during the study in the Chaparral area. *C. atrox* also occurs at the La Copita area, but none were observed during our tests.

First Field Study. The purpose of this study was to assess the repellency of scent gland secretions. Secretions from adult *C. atrox*, captured during March in Nolan and Brazos counties, Texas, were collected by manually pressing the base of the tail and allowing the scent gland exudates to flow into glass vials. The area of the cloaca from which scent gland secretions are released is shown in Figure 1. Two milliliters of dichloromethane (CH_2Cl_2) were added to each vial, and they were stored at -20°C . A metal spatula was used to apply 5–6 mg of the scent gland secretions or a commercially available coyote attractant, Carman's Canine Distant Call Lure (CDCL; Russ Carman, Milford, Pennsylvania), to cellulose sponges ($1 \times 1 \times 0.5$ cm) that had been immersed in water,

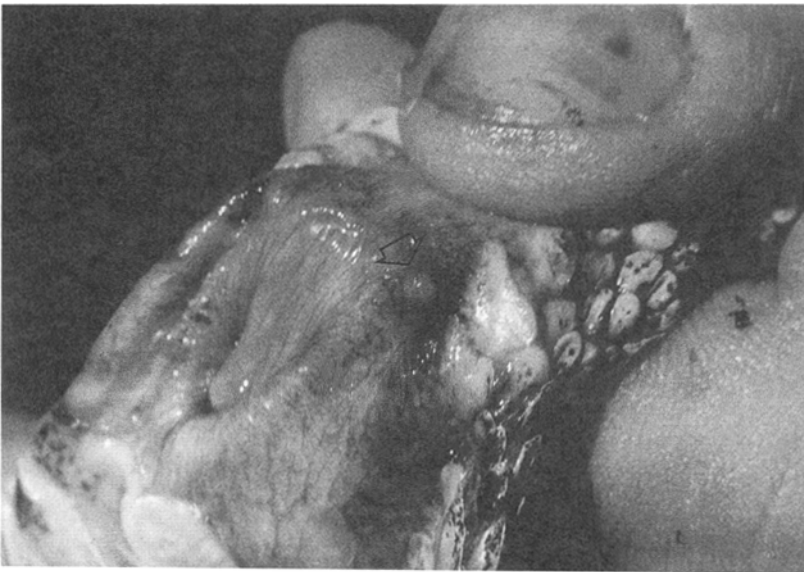


FIG. 1. Cloacal area of female *Crotalus atrox* showing papilla (arrow) from which scent gland secretions are discharged.

boiled for 20 min, and dried; control sponges received 0.3 ml of CH_2Cl_2 . The sponges were placed into perforated plastic capsules. One side of each capsule was pierced by a nail to secure it in the soil. All capsules were prepared within 24 hr of the first day of field presentations. They were kept in sealed glass jars until used.

Coyote responses to scent gland secretions were evaluated at the Chaparral area during July and August by using a scent station survey method, similar to that described in tests of coyote attractants (Turkowski et al., 1979, 1983). One hundred twenty scent stations were established, each within 2 m of roads extending 80 km into the study site, approximately 0.5 km apart on alternate sides of the road. Each station was comprised of two circular clearings (diameter = 1 m), 1–2 m apart, created by removing vegetation and sifting soil. A soft brush was used to smooth the soil surface so that tracks and other signs of animal activity could be discerned. A scent capsule was placed by hand (covered with a plastic glove) at the center of each circle. One clearing at each scent station received a capsule containing CDCL. Of the other clearings, half received capsules containing sponges treated with rattlesnake scent gland secretions; the other half received capsules containing sponges treated with CH_2Cl_2 . Capsules containing scent gland secretions and plain CH_2Cl_2 were placed at alternate scent stations.

Only one clearing per scent station typically is prepared during field surveys (e.g., Turkowski et al., 1979). We used two clearings during our first field study, one for the CDCL to attract coyotes. We expected coyotes to avoid scent gland secretions (cf., Price and LaPointe, 1981). We therefore deemed it necessary in this initial study to attract subjects to the proximity of scent gland samples so that an aversion could be distinguished from cases in which capsules were ignored. This experiment was not designed to evaluate the attractiveness of scent gland secretions, a property that was considered in subsequent tests.

Scent stations were inspected between 0700 and 1200 hr for evidence of animal activities occurring over the previous 24 hr during each of five consecutive days. The following information was scored from marks or deposits left within a 1 m diameter of the center of each circle: (1) visiting vertebrates, identified by their footprints, (2) defecation, (3) urination, as evidenced by small circular depressions (sometimes still moist when examined) in the soil, (4) rubbing and/or rolling, as evidenced by body depressions in soil and hairs deposited on ground or attached to scent capsules (Figure 2), (5) scratching and digging, and (6) pulling up capsules. Contaminated, damaged, or missing capsules were replaced each day, and the soil surface was smoothed by a brush. Scent stations on which tracks could not be identified, including those disturbed by wind or rain, were classified as "inoperable" and were reset with no data recorded from either clearing at the station.



FIG. 2. A plastic capsule containing *Crotalus atrox* scent gland secretions. The depression in the soil and hairs on the capsule indicate rubbing and rolling by a coyote.

Second Field Study. The purpose of this study was to assess the attractiveness of scent gland secretions. Scent gland secretions were obtained, stored at -20°C , and applied to sponges, as previously described; control sponges were untreated. Capsules containing untreated sponges or ones treated with scent gland secretions or CDCL were placed at stations at the La Copita area during March. Each scent station consisted of one circular clearing (diameter = 1 m) of smooth, sifted soil, located within 2 m and on alternate sides of the road. A total of 51 scent stations, 17 for each condition, were established. Stations were monitored for three consecutive days, where measures described for the first field study were scored.

Third Field Study. The purpose of this study was to determine whether behaviorally active chemicals from scent gland secretions can be extracted in an organic solvent and can survive rotary evaporation. Scent gland secretions were obtained and stored as described above. Approximately 80 ml of CH_2Cl_2 were added to the secretions, they were filtered, and the filtrate was placed on a rotary evaporator to remove the solvent. A 1.0 mg/ml solution of the residue of the scent gland secretions was prepared in CH_2Cl_2 , and 0.3 ml of this solution was applied to sponges; control sponges received 0.3 ml of CH_2Cl_2 .

Scent stations, comprised of one circular clearing each, were prepared at the Chaparral area during November. A capsule containing a sponge treated with scent gland secretion extract, CH_2Cl_2 , or CDCL was placed at each station. Sixty-six scent stations, 22 for each condition, were prepared and monitored for three consecutive days using measures described for the previous studies.

Domestic Dogs

Scent gland secretions, collected in vials as previously described, were transported on dry ice and stored at -70°C . A total of 30 ml of CH_2Cl_2 was added to the vials in 5-ml aliquots. After the addition of each aliquot, the vials were shaken for 1 min, a pipette was inserted beneath a layer of insoluble materials to draw off the CH_2Cl_2 , and the extract was filtered. The concentration of CH_2Cl_2 -soluble substances in the filtrate was determined by transferring 5 ml of the solution to a vial, immersing the vial in a water bath (45°C) under N_2 , and weighing the residue after drying for 24 hr in vacuo. A 5 mg/ml solution of secretions was prepared from the original (unevaporated) solution in an attempt to conserve volatile compounds.

Filter papers (diameter = 4.3 cm) each were treated with 0.5 ml of the scent gland solution; control papers received 0.5 ml of CH_2Cl_2 . The papers were air-dried for 5 min under a hood, placed into glass Petri dishes, covered, and kept at -70°C for several hours before use.

A total of 102 domestic dogs (*Canis familiaris*), pure and mixed breeds, was tested. They were housed individually in $1.2 \times 2.4 \times 2.7$ -m kennels, provided with water ad libitum, and fed between 1030 and 1200 hr. No information on the history of the subjects was available. All dogs had occupied their kennels at least 24 hr before testing.

Each dog was presented with snake-scented and control filter papers taped to the center of a 7×13 -cm metal plate. A plate with filter paper was inserted under the front of the kennel into a 15×15 -cm area designated by a chalk line drawn on the floor. The dogs' tendencies to enter the chalked area and paw at, sniff (by placing the snout within an estimated 5 cm from the paper), mouth (lick, bite, or eat the paper), and rub and roll on the paper were scored for 2 min to each condition. Two minutes elapsed between presentations, which were counterbalanced. All testing was done between 1200 and 1600 hr.

A separate test was done with 19 adult (> 10 months old) Staffordshire terriers, maintained in their home kennels ($1.5 \times 4.9 \times 1.8$ -m to $4.9 \times 7.6 \times 2.4$ -m) for at least three months. The sex of subjects was recorded. Dogs were presented with filter papers treated with plain CH_2Cl_2 and CH_2Cl_2 extracts of secretions of rattlesnake scent glands or American alligator (*Alligator mississippiensis*) paracloacal glands. Paracloacal glands are paired exocrine organs (not homologous with scent glands) in the cloacal walls of crocodylians, and are suspected of containing pheromones. An analysis of these secretions indicates a variety of esters, 3-methylbutanoates, free fatty acids, and other compounds (Weldon et al., 1988). The paracloacal gland secretions, collected from adult male alligators during September in Port Arthur, Texas, were included here as an additional control condition.

Both rattlesnake and alligator secretions were stored in vials with several ml of CH_2Cl_2 . They were filtered after adding 30 ml in 5-ml aliquots of CH_2Cl_2 to the samples, as described above.

Aliquots of snake scent gland and paracloacal gland extracts were weighed, and the solutions from which they were derived were dissolved in CH_2Cl_2 to give 6.3 mg/ml solutions, 0.5 ml of which was applied to filter papers. Control papers received 0.5 ml of CH_2Cl_2 . The papers were air-dried for at least 5 min before being presented to the dogs.

The filter papers were taped to metal plates and placed in the dogs' home kennels by their owners. Each dog was presented with control, snake-scented, and alligator-scented papers for 2 min each in a Latin-square design. Sniffing, mouthing, pawing, rubbing and rolling, and urination postures (leg-lifting and squatting) were scored; since it was not always clear whether micturition occurred, we conservatively scored the occurrence of only the associated squatting or leg-lifting postures. One to two minutes elapsed between presentations. Tests were run between 1000 and 1700 hr, before the dogs were fed.

RESULTS

Coyotes

First Field Study. The chi-square test was used in all field data analyses. Omitting those scent stations classified as inoperable, 756 observations remained for analysis. The most common vertebrates visiting scent stations over the five-day test period are listed in Table 1. Visitation rates to scent stations by all species combined did not differ significantly ($P > 0.40$) from day to day. Visitation rates of coyotes for days 1–5 were 21%, 36%, 36%, 27%, and 32%, respectively ($P > 0.50$).

Coyotes visited 33% of the scent stations in the Chaparral area. There was

TABLE 1. FREQUENCY OF RESPONSE (%) BY COYOTES AND OTHER MAMMALS IN SOUTH TEXAS TO SCENT STATIONS WITH RATTLESNAKE SCENT GLAND SECRETIONS, DICHLOROMETHANE, AND COYOTE ATTRACTANT (CARMAN'S CANINE DISTANT CALL LURE)

Animals and behavioral response	Response (%)		
	Rattlesnake secretion ($N = 193$)	Dichloromethane ($N = 185$)	CDCL ($N = 378$) ^a
Coyote			
Visits	30.6	33.5	33.1
Urination	2.6	4.9	3.2
Defecation	3.1	0.0	1.3
Groundscratching	1.6	0.0	1.1
Capsule pulling	22.3	20.5	25.7
Rubbing/rolling	9.3 ^b	2.2 ^a	13.6 ^b
Raccoon			
Visits	4.1	2.7	4.5
Striped skunk			
Visits	1.6	2.2	2.4
Javelina			
Visits	3.1	3.7	3.5
Rodents			
Visits	80.3	73.5	78.3
Lagomorphs			
Visits	8.8	9.2	6.4

^a CDCL was present at every station so that there were twice as many observations as for the other two treatments.

^b Means with different letters are significantly ($P < 0.05$) different.

no evidence on the basis of visitations alone that coyotes discriminated between treatments ($P > 0.50$), indicating their failure to avoid rattlesnake scent gland chemicals (Table 1). A significantly greater frequency of rubbing and rolling occurred at snake secretion-scented stations than occurred at control stations ($P < 0.01$). No significant differences were detected in the frequency with which snake secretion-scented or control capsules were disturbed ($P > 0.60$), or in urinations that occurred at snake secretion-scented or control stations ($P > 0.20$). Defecation and ground-scratching occurred at frequencies too low to permit analysis.

A comparison of coyotes' responses to capsules treated with snake scent gland secretions and coyote attractant, CDCL, on adjoining circles indicated no significant differences between these conditions with respect to rates of visitation ($P > 0.50$), capsule-pulling ($P > 0.30$), urination ($P > 0.10$), defecation ($P > 0.10$), or ground-scratching ($P > 0.60$). Coyotes rubbed and rolled at 9% of snake secretion-scented stations, but this response did not differ significantly ($P > 0.10$) from the rate at which this occurred (14%) at CDCL stations. Coyotes rubbed and rolled during 30% of their visits to snake secretion-scented stations; they did so at 41% of the CDCL stations visited.

Other taxa, particularly rodents, were frequent visitors at scent stations (Table 1). There were no differences in visitation rates at snake secretion-scented and control stations for rodents ($P = 0.14$) or lagomorphs ($P = 0.89$), indicating a failure to avoid rattlesnake scent gland chemicals. We also failed to detect significant differences in visitations at snake secretion-scented and control stations for raccoons ($P > 0.60$), striped skunks ($P > 0.20$), and javelina ($P > 0.50$), but visitation rates for these taxa were low.

No behavioral responses other than capsule-pulling were recorded for animals other than coyotes, and in many of these instances the capsules appeared to have been kicked or trampled inadvertently. Almost all of the capsules remained within the 1 m circle where species other than coyotes were involved. Coyotes typically carried the capsule at least several meters. There were 18 instances in which the capsules with any of the three stimuli were pulled by all other species combined, for a frequency of 2%, in contrast to 24% for coyotes.

Second Field Study. After eliminating inoperable stations at La Copita, a total of 146 observations remained for analysis (Table 2). Visitation rates of coyotes for days 1–3 were 22%, 10%, and 14%, respectively.

No significant differences were detected in the frequency with which coyotes visited snake secretion- and CDCL-treated stations ($P > 0.05$), but visits to both of these were higher than to the control stations ($P = 0.05$). Nearly all capsule-pulling was directed at scent gland secretions and CDCL; the difference between these was slight. Rubbing and rolling was not observed at control stations, but it occurred at 10% and 11% of the snake secretion- and CDCL-treated

TABLE 2. FREQUENCY OF RESPONSE (%) BY COYOTES AND OTHER MAMMALS AT LA COPITA RESEARCH AREA TO SCENT STATIONS WITH RATTLESNAKE SCENT GLAND SECRETIONS, UNTREATED CONTROL, AND COYOTE ATTRACTANT (CARMAN'S CANINE DISTANT CALL LURE)

Animals and behavioral response	Response (%)		
	Rattlesnake secretion (<i>N</i> = 50)	Untreated control (<i>N</i> = 51)	CDCL (<i>N</i> = 45)
Coyote			
Visits	22.0 ^a	5.8 ^b	17.8 ^a
Urination	2.0	0.0	0.0
Defecation	0.0	0.0	0.0
Groundscratching	0.0	0.0	0.0
Capsule pulling	14.0	1.9	13.3
Rubbing/rolling	10.0	0.0	11.1
Raccoon			
Visits	20.0	5.8	17.8
Striped skunk			
Visits	0.0	0.0	2.2
Javelina			
Visits	10.0	3.9	6.6
Lagomorphs			
Visits	44.0	35.3	53.3

^aMeans with different letters are significantly ($P < 0.05$) different.

stations, respectively. Coyotes rubbed and rolled during 71% and 83% of their visits to stations with scent gland secretions and CDCL, respectively.

Evidence of visits by other vertebrates was indicated at the scent stations (Table 2). No significant differences in visits were noted where the sample sizes for these taxa were adequate for chi-square comparisons.

Third Field Study. After eliminating inoperable stations at the Chaparral area, a total of 196 observations remained for analysis (Table 3). Visitation rates of coyotes for days 1-3 were 26%, 21%, and 38%, respectively.

The CDCL stations were visited by coyotes more frequently than were either snake secretion-scented or CH₂Cl₂-treated stations ($P < 0.05$). Capsule-pulling and rubbing and rolling were the only behavioral measures recorded in numbers adequate for analysis. Evidence of both responses was observed more frequently at the CDCL stations than at those treated with either scent gland secretions or CH₂Cl₂ ($P < 0.05$). The frequency with which snake secretion-scented capsules were pulled did not differ significantly ($P < 0.05$) from that

TABLE 3. FREQUENCY OF RESPONSE (%) BY COYOTES AND OTHER MAMMALS AT CHAPARRAL WILDLIFE MANAGEMENT AREA TO SCENT STATIONS WITH EXTRACT OF RATTLESNAKE SCENT GLANDS SECRETIONS (AFTER ROTARY EVAPORATION), DICHLOROMETHANE, AND COYOTE ATTRACTANT (CARMAN'S CANINE DISTANT CALL LURE)

Animals and behavioral response	Response (%)		
	Rattlesnake secretion (N = 66)	Dichloromethane (N = 66)	CDCL (N = 64)
Coyote			
Visits	19.7 ^a	21.2 ^a	45.3 ^b
Urination	3.0	7.5	10.9
Defecation	0.0	0.0	3.1
Groundscratching	1.5	0.0	9.3
Capsule pulling	13.6 ^a	16.6 ^a	34.4 ^b
Rubbing/rolling	9.0 ^a	0.0 ^a	39.0 ^b
Raccoon			
Visits	10.6	6.0	17.1
Striped skunk			
Visits	1.5	0.0	0.0
Javelina			
Visits	6.0	3.0	4.7
Rodents			
Visits	60.6	63.6	59.4
Lagomorphs			
Visits	40.9 ^a	40.0 ^a	21.9 ^b

^a Means with different letters are significantly ($P < 0.05$) different.

observed with CH_2Cl_2 -treated capsules; however, no rubbing and rolling was observed in response to CH_2Cl_2 -treated capsules.

Raccoons visited 11% of the scent stations, most frequently those containing CDCL ($P > 0.10$) (Table 3). Lagomorphs and rodents were frequent visitors, but only the former exhibited differences in visitation rates for the three conditions, visiting CDCL stations less frequently than either snake secretion- or CH_2Cl_2 -scented stations ($P < 0.05$). Other species visited stations too infrequently for chi-square analyses.

Domestic Dogs

Of the 102 dogs tested in the kennel survey, 77 exhibited one or more of the scored behaviors during either control or experimental presentations. Only data from these responding individuals are presented and subjected to statistical

TABLE 4. RESPONSES OF 77 DOMESTIC DOGS, PURE AND MIXED BREEDS, DURING 2-MIN PRESENTATIONS OF FILTER PAPERS TREATED WITH DICHLOROMETHANE AND DICHLOROMETHANE EXTRACT OF RATTLESNAKE SCENT GLAND SECRETIONS

Treatment	\bar{X} times entered square (No. of dogs)	No. dogs pawing	\bar{X} sniffing bouts (No. of dogs)	No. dogs mouthing	No. dogs rubbing and rolling
Dichloromethane	1.5 (59)	14	1.3 (66)	15	4
Scent gland	1.9 (66)	15	1.8 (63)	26	8

analyses (Table 4). The chi-square test indicates that dogs mouthed filter papers treated with the CH_2Cl_2 extract of scent gland secretions more than controls ($P < 0.05$). No other significant differences between conditions were detected.

Of the 19 Staffordshire terriers tested, 12 exhibited one or more of the scored behaviors during either control or experimental presentations. Only data from these responding individuals are presented here and subjected to statistical analyses. Rubbing and rolling was observed during 0, 1, and 3 sessions, and urination postures during 1, 2, and 6 sessions with CH_2Cl_2 , paracloacal gland secretions, and scent gland secretions, respectively. All urination postures, except one in which a female urinated directly on an alligator-scented paper, were exhibited by males. No significant differences were detected between conditions for rubbing and rolling. The Fisher exact-probability test, which was used due to the small sample size, indicates that urination postures occurred during significantly more sessions with scent gland secretions than with CH_2Cl_2 ($P < 0.05$), but responses to snake- and alligator-treated papers did not differ significantly ($0.10 > P > 0.05$).

DISCUSSION

The reactions of predatory mammals to snake scent gland secretions were observed by Price and LaPointe (1981), who presented desert kingsnake (*Lampropeltis getulus splendida*) exudates on food to badgers, coyotes, and other North American carnivores. The coyotes' responses were not specifically described, but Price and LaPointe (1981) characterized the overall reactions to scent gland secretions by the carnivores they examined as "aversionary."

The results of our first two field experiments indicate that coyotes rubbed and rolled on rattlesnake scent gland secretions, as they did on the coyote attractant, CDCL. In our third field test, however, rubbing and rolling did not occur to scent gland extracts at a rate significantly different from that to CH_2Cl_2 ,

although this behavior was indicated at a significantly higher rate to CDCL. This suggests that the chemicals eliciting rubbing and rolling are not retained (in sufficient quantities) following our methods of extraction, rotary evaporation, and field presentation. Domestic dogs in both our kennel tests also failed to rub and roll on CH_2Cl_2 extracts of scent gland secretions significantly more often than on control papers. Scent gland compounds insoluble in CH_2Cl_2 , i.e., glyco- or mucoproteins and acid mucopolysaccharides (Whiting, 1969; Blum et al., 1971), likely were denatured or removed by our extraction methods. These substances may provide a matrix for the retention of volatile, behaviorally active chemicals.

Bullard (1982) states that most, if not all, canids rub and roll on putrefying materials. Fox and Cohen (1977) report rubbing and rolling by a variety of canids to natural and synthetic substances, including skatole and propionic acid. Although sterols and fatty acids have been indicated in snake scent glands (Blum et al., 1971; Simpson et al., 1988), Oldak (1976) failed to detect these or other nonpolar lipids in his thin-layer chromatograms of crotaline scent gland secretions, including those of a rattlesnake. Razakov and Sadykov (1986) identified fatty acids from the scent glands of the mamushi (*Agkistrodon halys*), indicating that these compounds are present in at least some crotalines. Chemicals in the secretions of *C. atrox* need to be identified and tested as synthetic analogs to determine those that elicit canid responses.

In our study, Staffordshire terriers exhibited urination postures more frequently to rattlesnake secretion extracts than to CH_2Cl_2 , but snake- and alligator-treated papers did not differ significantly in their effects. Urination (or urination posturing) was not scored during our first kennel survey, and it occurred at too low a frequency for statistical analysis during our field studies. The tendency of canids to urinate on conspecific urine deposits often is interpreted as a form of scent marking. Urination also may be elicited by "novel" odors, such as those arising from other species' feces (Fox and Cohen, 1977). Kool (1981) noted that the dingo (*C. familiaris*) urinated on a turtle's (*Chelodina longicollis*) Rathke's gland secretions, which are hypothesized to deter predators. In Kool's study, as with ours, the adaptive significance of eliciting urination in canids is not obvious.

Neither are the aversive properties of scent gland secretions evident from our results. In the first field study, designed to measure the potential repellency of snake scent gland secretions, coyotes visited capsules containing these exudates at a rate that was not significantly different from that to capsules containing CH_2Cl_2 . In the second field study, designed to measure the potential attractiveness of rattlesnake scent gland secretions, coyotes visited stations containing secretion extracts significantly more often than those containing untreated capsules and at a rate not significantly different from that to CDCL. This is worth noting since CDCL has been developed specifically as a lure for these

carnivores and is one of the most effective commercial attractants available (Turkowski et al., 1983).

We did not test the palatability of untreated scent gland secretions in our experiments, thus, we cannot relate our results directly to those of Price and LaPointe (1981). However, the tendency of dogs to mouth snake secretion-scented filter papers and that of coyotes in the field to pull up (presumably by mouth) snake secretion-scented capsules does not support the hypothesis that these materials are distasteful. Palatability studies using whole, untreated scent gland substances are needed.

Scent gland secretions could interfere with predation in ways other than by having aversive effects. Rubbing and rolling, for example, could distract canids from predatory attack. Even the elicitation of mouthing could be advantageous for snakes if scent gland chemicals direct attacks toward the tail, and away from their head and trunk, in a way analogous to that in which visually directed predators are diverted by snake tail displays (Greene, 1987). Insight into the mechanisms by which scent gland secretions benefit snakes could be obtained by observing interactions between snakes and predators and, perhaps, by comparing the survivorship of intact snakes with that of those from which these glands have been surgically removed.

Scent glands are homologous among extant snakes. Since the common ancestor of Recent snakes lived during the Upper Cretaceous (Rage, 1987) this can be considered a minimum age for these organs. Carnivores did not appear until during the Tertiary (Ewer, 1973). Given this chronology, it is clear that snake scent glands did not evolve as a response to these mammalian predators. The antipredator function(s) of the exudates produced in these organs needs to be tested on other taxa, e.g., varanid lizards and crocodylians.

Functions and effects other than the production of predator repellents have been attributed to scent glands, including that of pheromone release. Although experiments suggest that blindsnake (*Leptotyphlops dulcis*) scent gland chemicals attract conspecifics (Watkins et al., 1969), attempts to elicit trail-following with these substances in other snakes have failed (reviewed in Ford, 1986). Graves and Duvall (1988) report that airborne presentations of scent gland chemicals from the prairie rattlesnake (*C. viridus*) elicit increased heart rates in conspecifics, and they suggest, following Brisbin's (1968) observations on a common kingsnake (*Lampropeltis getulus*), that these organs release alarm pheromones. Even so, whether scent gland chemicals function primarily to alert conspecifics is uncertain since alarm properties often are derived secondarily from compounds released during predatory attack (Weldon, 1983).

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AGGREGATION IN YELLOW MEALWORMS, *Tenebrio molitor* L. (COLEOPTERA: TENEBRIONIDAE) LARVAE
I. Individual and Group Attraction to Frass
and Isolation of an Aggregant

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Abstract—Late-instar larval *Tenebrio molitor* L. were found to be attracted to aqueous extracts of conspecific larval frass. The attraction was evident at both the individual and group level. The attraction of larval groups to frass indicated the possibility of an aggregation pheromone that would be chemically distinct in the mealworm environment. Chemical analysis of short carbon chain acids present in both the mealworm frass and the diet indicated that lactic acid was present in the mealworm frass only. Acetic acid was identified in both the diet and the larval frass. Larvae aggregated on filter papers treated with aqueous frass extracts that had been dried and also on those freshly wetted. The larvae also aggregated on dried or freshly wetted papers treated with lactic acid, but failed to aggregate on freshly wetted papers or dried papers treated with acetic acid. The role of excreted lactic acid as a discriminant of already infested and, therefore, safer environmental regions is discussed.

Key Words—Aggregation, lactic acid, yellow mealworm, *Tenebrio molitor*, frass, acetic acid, Coleoptera, Tenebrionidae, attraction.

INTRODUCTION

The yellow mealworm, *Tenebrio molitor* L. is a cosmopolitan stored-products pest of economic importance with infestations generally originating in damp, dark places where cereals may be decaying (Cotton, 1963). The presence of the

conspicuous larvae in a stored-products mass is an indication of poor storage sanitation (Sinha and Watters, 1985). Pheromone communication among *Tenebrio* adults has been studied (Tschinkel et al., 1967; Happ, 1969; Tanaka et al., 1986). McFarlane and Alli (1986) have shown DL-lactic acid to be an excreted aggregant of larval *Blattella germanica*. In this article, aggregation of larval *Tenebrio molitor* on frass-treated substrates is evaluated. Chemical analysis of diet and frass for short carbon chain acids indicated candidate chemicals that may be used by yellow mealworms to analyze substrates for suitability for aggregate formation. These were tested in a behavioral bioassay. The interaction between water and pheromone found in lactic acid-induced larval aggregation in the German cockroach (McFarlane and Alli, 1986) was also evaluated for *Tenebrio*. This article is the first report of a larval aggregation pheromone in *Tenebrio*. This pheromone was also evaluated for epideictic activity (Wynn-Edwards, 1962; Prokopy, 1981).

The objectives of the following experiments were to evaluate a broad range of concentrations of pheromone for behavioral activity and to observe the nature of the response of the insect to these chemicals during a limited period of presentation in a behavioral bioassay. The possible role of this pheromone in population orientation is discussed.

METHODS AND MATERIALS

Rearing. Yellow mealworms, *Tenebrio molitor* L., were raised on a diet of wheat bran, whole wheat flour, and brewer's yeast (50:45:5 w/w) at $25 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ relative humidity and a photoperiodic regime of 14:10 light-dark. The density of all stages per filled 4.55 liter glass culture jar was not allowed to exceed 250 individuals.

Collection of Frass and Diet. Larvae (> 100 mg) were removed from the culture and individually placed in 20-ml glass jars for 24 hr. Insects were then removed and the collected frass was immediately frozen at -40°C . Frass from any molting larvae was discarded to avoid contamination by the cuticle. Freshly prepared diet was also frozen at $-40 \approx \text{C}$.

Methods of Acid Determination in Frass and Diet. Frass (380 mg) was ground with a mortar and pestle and placed in a 40-ml centrifuge tube. A quantity (20 ml) of deionized distilled water was added to the test tube and the mixture was agitated (1 min) with a vortex mixer and allowed to stand 2 min. This agitation procedure was repeated three times. The resulting mixture was centrifuged (200 g, 10 min), and the supernatant was filtered into a volumetric flask (25 ml). The same procedure was followed for the diet sample (500 mg).

For acid determination by gas chromatographic analysis, the acids were

converted to benzyl ester derivatives (Jones and Kay, 1976). Method II of McFarlane and Alli (1986) indicates column specifications and conditions of gas chromatographic analysis and mass spectrometric confirmation. The method of frass analysis for D- and L-lactic acid was that of McFarlane and Alli (1986).

Bioassay Arena. The arena used was similar to that of McFarlane *et al.* (1983). Experiments were conducted at room temperature and in arenas kept in darkness. Two 14×3.8 -cm strips of Whatman No. 1 filter paper folded three times were placed on edge and evenly spaced in a 19.2-cm-diam. glass culture dish. Test papers were maintained upright by clamping the upper edges of the test paper with two clamp-style hairpins suspended from a copper wire extending across the arena and attached to either side by clothespins.

Testing for Frass Attraction. Frozen frass was ground with a mortar and pestle and added to 5 ml of deionized, distilled water. The resulting mixture was agitated in a vortex mixer for 2 min and immediately subjected to Buchner filtration through a Whatman No. 1 filter disk. An aliquot (0.68 ml) of the filtered liquid was immediately applied to a test strip. A control strip was wetted with deionized, distilled water. Other frass test strips were similarly prepared and allowed to air dry 1.5 hr prior to testing. A yellow mealworm (140–160 mg) was introduced into the center of the arena to test for individual attraction; 20 such larvae were used to test group responses. The filter papers were examined at 0.5-hr intervals after the initiation of the trial. The criteria for aggregation/attraction included larvae in full contact with the test strip or those insects contacting the test strip with their antennae, mouthparts or legs, but not actually upon the paper. Wandering, inactive, or clustering larvae not touching the test paper were considered a covariable for analysis comparing mean numbers of larvae on treatment papers in separate group trials. The dominant response of tested mealworms was to come to rest on or in contact with a test strip. Each trial was terminated after 5 hr. All group trials were replicated 18 times.

Testing for Lactic, Acetic, and Selected Acid Attraction. DL-Lactic acid (Fisher Scientific Company, Montreal, Canada) and acetic acid (Anachemia Limited, Montreal, Canada) were tested by making serial dilutions in deionized distilled water and treating a filter paper strip with 0.68 ml of solution. Dried and wetted test papers were prepared and tested using the method described in the frass aggregation bioassay. Grouped response trials using citric and phosphoric acids (Anachemia), with pK_a s similar to lactic acid, also followed this protocol.

Trials to Evaluate Larval Exploration and Arrest. To determine if either lactic acid or frass were responsible for either larval attraction or full arrest, groups of larvae and individual larvae were subjected to the described bioassay and directly observed. Data collected were the choice of the first two insects in each group trial and whether these first two responding larvae remained on the

test strip for at least 300 sec. Replication was conducted as soon as possible after the previous trial was completed ($\leq 12/\text{day}$), 36 trials in total. Individual larvae were observed to determine the significance of their initial choice and tendency to remain on the chosen substrate for at least 300 sec. This was undertaken to avoid bias possible by scrutinizing data collected at preset times only and also to determine if, in the grouped trials, later larval response is biased by the presence of those first responding individuals.

Statistical Analysis. Aggregation bioassay data were subject to analysis of covariance. Comparisons between distributions on the two papers used the mean number in each location through the period from apparent stabilization (90 min) to the end of the experiment (300 min) (Ross and Tignor, 1986) using Tukey's studentized range (HSD) test on the means with $\alpha = 0.05$. Comparisons between test chemical types were based on a *posteriori* analysis of mean larval response to the treated chemical strips (SAS, 1982). Chi-square analyses were used to test for significant differences in control vs. control trials (groups and individuals) and all treatment vs. control individual trials. Chi-square analyses were also used to determine if the choices of the first two responding larvae were significantly different in group treatment vs. control tests and to determine if there was a difference in the tendency to arrest of insects responding to either treatment or control papers during their initial exploration of the arena.

RESULTS

Analyses of frass indicated lactic acid at 0.24 ± 0.01 g/100 g frass and acetic acid at 0.25 ± 0.01 g/100 g frass. However, analyses of the diet indicated that although acetic acid was present (0.023 ± 0.004 g/100 g diet), lactic acid is not present in the mealworm diet. The lactic acid isomeric analysis gave the following results: 0.098% D-lactic acid and 0.087% L-lactic acid per frass sample (frass was 0.185% lactic acid). Dry weight determination indicates fresh frass has a 10.2% moisture content and freshly prepared diet has a 9.8% moisture content.

All tested concentrations of frass resulted in aggregation of the larvae, with the highest numbers responding to the highest concentration. Aggregation always occurred on papers treated with frass extracts whether they were presented wet or dry (Table 1). Papers treated with 0.68 ml (the minimum amount of liquid required to fully wet the filter paper) of 10^{-1} M lactic acid, both freshly wetted and dried, were found to be repellent. Papers treated with 0.68 ml of 10^{-2} – 10^{-4} M lactic acid and dried were most attractive, with 10^{-2} M freshly wetted being slightly less attractive. Aliquots of lactic acid at concentrations below 10^{-4} M showed no effect (Table 2).

TABLE 1. RESULTS OF AGGREGATION TRIALS USING FRASS EXTRACTS^a

Chemical	Concentration (mg/ml H ₂ O)	Treated ^b	Control ^c
Frass (dry)	50	13.08 ± 0.32a	2.17 ± 0.36*
Frass (wet)	40	12.55 ± 0.37a	4.53 ± 0.41*
Frass (dry)	30	10.92 ± 0.54ab	4.95 ± 0.54*
Frass (wet)	20	10.67 ± 0.58ab	6.28 ± 0.34**

^aBased on 18 replicates of 20 larvae each. Raw data based on counts from time of approximate stabilization to trial end (1.5–5.0 hr).

^bMeans followed by the same letter not significantly different ($P \leq 0.05$). Units are mean number of larvae.

^c*Treated significantly greater than control at $P \leq 0.001$. **Treated significantly greater than control at $P \leq 0.005$.

Acetic acid was found to have no effect on the larvae at any concentration, regardless of the method of presentation (Table 3).

All control vs. control trials indicated no significant differences in preference for substrate (data not shown). Geomagnetic bias is unlikely, therefore.

Figure 1 illustrates the insect response to dried papers treated with 0.68 ml

TABLE 2. RESULTS OF AGGREGATION TRIALS USING LACTIC ACID SOLUTIONS^a

Chemical and Concentration	Treated ^b	Control ^c
Lactic acid (dry)		
10 ⁻¹ M	4.97 ± 0.61c	11.26 ± 0.43***
10 ⁻² M	11.92 ± 0.51a	5.74 ± 0.21*
10 ⁻³ M	12.94 ± 0.38a	3.27 ± 0.25*
10 ⁻⁴ M	11.33 ± 0.47a	6.70 ± 0.23**
10 ⁻⁵ M	8.47 ± 0.38b	9.82 ± 0.19 NS
10 ⁻⁶ M	9.10 ± 0.44ab	8.32 ± 0.22 NS
Lactic acid (wet)		
10 ⁻¹ M	5.46 ± 0.65c	10.97 ± 0.43***
10 ⁻² M	9.38 ± 0.66ab	6.41 ± 0.44**

^aBased on 18 replicates of 20 larvae each. Raw data based on counts from time of approximate stabilization to trial end (1.5–5.0 hr).

^bMeans followed by the same letter not significantly different ($P \leq 0.05$). Units are mean number of larvae.

^c*Treated significantly greater than control at $P \leq 0.001$. **Treated significantly greater than control at $P \leq 0.005$. ***Control significantly greater than treated at $P < 0.001$. NS: No significant difference between treated and control.

TABLE 3. RESULTS OF AGGREGATION TRIALS USING ACETIC ACID SOLUTIONS^a

Chemical	Concentration	Treated ^b	Control
Acetic acid (wet)	10 ⁻¹ M	8.17 ± 0.28b	7.41 ± 0.35 NS ^c
Acetic acid (dry)	10 ⁻² M	7.94 ± 0.36b	6.97 ± 0.48 NS
Acetic acid (wet)	10 ⁻³ M	8.61 ± 0.39b	7.77 ± 0.39 NS
Acetic acid (dry)	10 ⁻⁴ M	9.04 ± 0.52ab	8.74 ± 0.68NS

^aBased on 18 replicates of 20 larvae each. Raw data based on counts from time of approximate stabilization to trial end (1.5-5.0 hr).

^bMeans followed by the same letter not significantly different ($P < 0.05$). Units are mean number of larvae.

^cNS: No significant difference between treated and control.

of the highest concentration of aqueous frass during the aggregation bioassay. Figure 2 illustrates the insect response to dried papers treated with 0.68 ml of 10⁻² M lactic acid during the bioassay. The two graphs show similar overall responses (mean number of larvae on treated paper from 1.5 to 5.0 hr), but early in the frass extract trials there were significantly fewer insects at rest on the control papers. The concentration of lactic acid on the frass-treated paper (Figure 1) is equivalent to that on filter paper treated with a 0.68-ml aliquot containing lactic acid between 10⁻² M and 10⁻³ M. The time required for stabilization in trials which indicated successful aggregation (Figures 1 and 2),

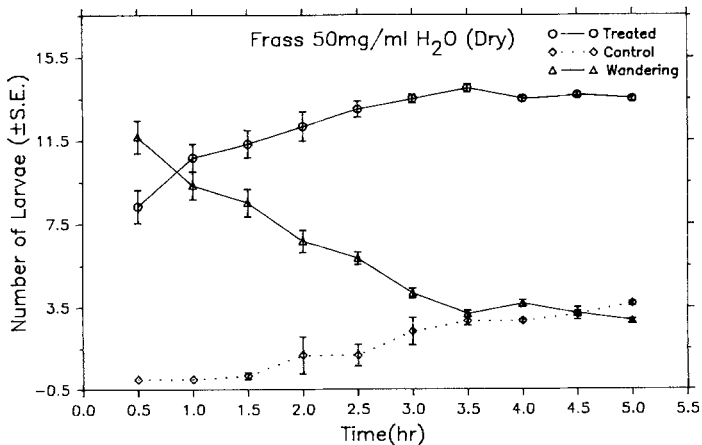


FIG. 1. Mean number of mealworm larvae (±SE) on papers treated with 50 mg frass/ml H₂O and dried. Based on 18 replicates of 20 larvae each. Wandering indicates insects not on filter papers.

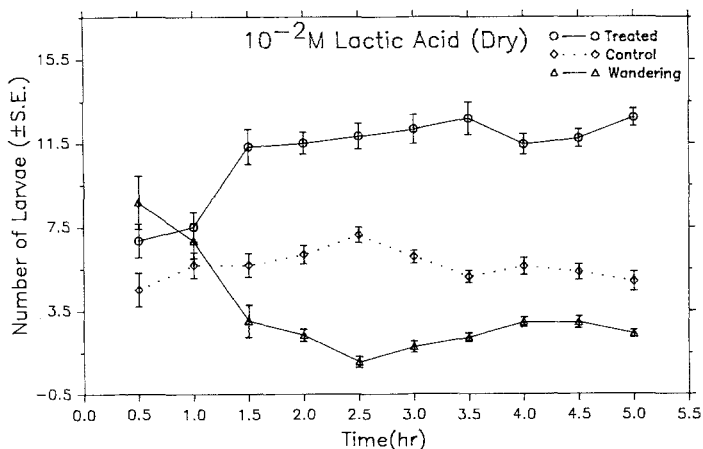


FIG. 2. Mean number of mealworm larvae (\pm SE) on papers treated with 10^{-2} M lactic acid and dried. Based on 18 replicates of 20 larvae each. Wandering indicates insects not on filter papers.

was approximately 1.5 hr, at which point no further large increases in numbers on either treated or control test strips occurred. Variations occurring after stabilization generally resulted from insects falling from the filter paper into the arena or larvae that were initially inactive becoming active and responding to the filter papers at a later trial interval. In trials where repellency or an indifferent response was indicated, stabilization occurred more gradually, but trends towards the final response can be seen at 1.5 hr. Figure 3 illustrates the insect response to repellent 10^{-1} M lactic acid (wet). There was a significantly higher number of larvae on the control paper at 1.5 hr but the difference became more obvious at 2 hr. The response of the larvae to an indifferent chemical stimulus (10^{-3} M acetic acid) is illustrated in Figure 4. In this case, stabilization is gradual, with an increase in wandering insects occurring between 3.0 and 4.5 hr. This indicates that prolonged grouped stability was less likely in trials where no chemical message was perceived.

Aggregation bioassays using inorganic and organic acids with pK_a s similar to DL-lactic acid indicated that both citric and phosphoric acid were repellent to groups of larvae when presented wetted on filter paper in aqueous solution at concentrations of 10^{-1} M and 10^{-2} M. Lower concentrations produced no effect (data not shown).

Tests indicated individual larvae were attracted to frass and lactic acid in the same manner as grouped larvae with counts taken at predetermined intervals. Table 4 gives the chi-square values for a selected frass solution and 10^{-3} M lactic acid at 2- and 4-hr intervals.

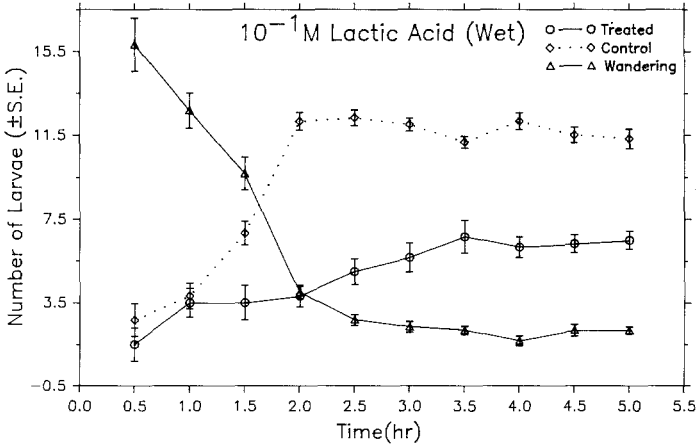


FIG. 3. Mean number of mealworm larvae (\pm SE) on papers wetted with 10^{-1} M lactic acid. Based on 18 replicates of 20 larvae each. Wandering indicates insects not on filter papers.

Evaluation of first responding individuals (group tested) indicated that overall response of the larvae to treatment or control was not determined by the presence of other larvae (choice of first larvae reaching either test or control strips indicated no significant difference in preference), nor did first responding

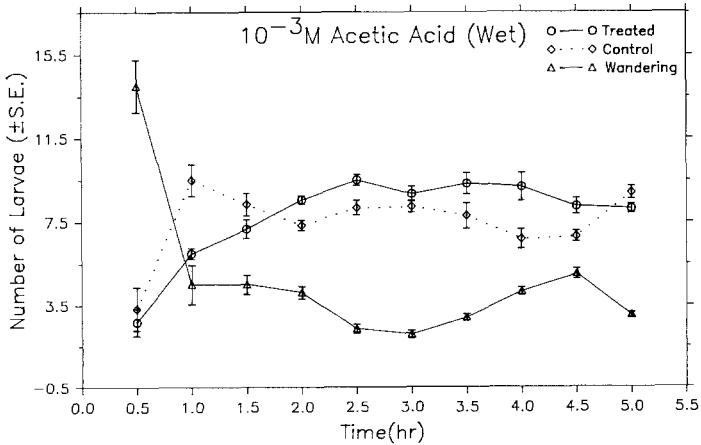


FIG. 4. Mean number of mealworm larvae (\pm SE) on papers wetted with 10^{-3} M acetic acid. Based on 18 replicates of 20 larvae each. Wandering indicates insects not on filter papers.

TABLE 4. RESULTS OF ATTRACTANCY TRIALS USING INDIVIDUAL *Tenebrio molitor* LARVAE

Chemical	Time (hr)	Treated ^a	Control ^b
Lactic acid (10 ⁻³ M) (dry)	2	30	13*
	4	38	15**
Frass 30 mg/ml (wet)	2	32	12**
	4	41	14***

^aBased on five trials of 12 replicates each.

^b*Treated significantly higher than control at $P \leq 0.025$. **Treated significantly higher than control at $P \leq 0.005$. ***Treated significantly higher than control at $P \leq 0.001$.

larvae remain on either treatment or control filter paper for the 300-sec interval in significantly different numbers. In fact, fewer than 60% of the first-responding treatment or control larvae remained for this amount of time. Individual larvae did not show any significant differences in their first choice, but those selecting frass or lactic acid tended to remain longer on the paper; however, 31% of the observed individuals initially selecting test chemical stimuli left the papers only to return subsequently (data not shown).

DISCUSSION

Both dried and wetted lactic acid test strips are the preferred substrates for the larvae of *T. molitor* at 10⁻²-10⁻⁴ M concentrations. This range approximates the amount found in the fresh larval frass. Other chemicals present in the frass exert different behavioral effects when tested in isolation, but their efficacy is completely masked by lactic acid when tested along with lactic acid at the biological concentration (Weaver *et al.*, 1990). The frass in entirety seems to be exclusively attractive, therefore.

The larval frass has a water content of 10.2%. This indicates that the lactic acid in the frass is unlikely to be in solution, but more correctly, is substrate-associated. This may, in part, account for the fact that the required interaction between pheromone and water (larvae only responding to papers wetted with aqueous acid solution) found in other studies (McFarlane *et al.*, 1983; McFarlane and Alli, 1986; 1987) is not required for *Tenebrio*. The surrounding diet has a 9.8% water content, so environmental stimuli are unlikely to require water as a necessary component. Mealworms are also hygrotaxic in the stored product environment when a water source is available; ecologically they prefer damp surroundings. It is possible that they display two similar patterns of response to frass stimuli. In one case they will aggregate in response to frass in

the appropriate concentration in a dry but species-marked environmental region. In the other case they may respond to moisture in a damp region and may use the presence of lactic acid for the same purpose, to cluster. Group hygrotaxis is a form of aggregation in any case; the important distinction is that damp regions also containing frass could provide information on earlier infestation.

The trials utilizing acids with pK^a 's similar to lactic acid indicate the degree of dissociation of the acid is not responsible for aggregation by these larvae (data not shown). This is unlikely to be a factor in an environment lacking free liquid to act as a solvent.

These components of the aggregation behavior bring to light an important issue. It is necessary to carefully consider all data provided by a particular bioassay. In the case of these trials, it appears that these insects show classical attraction to lactic acid. However, the length of time required for response to occur and the relatively consistent variation of insects that do not respond to acetic acid indicates that these larvae respond to their substrate by contact sampling it in a relatively regular manner (Weaver and McFarlane, 1989). The response may be similar to that described by Burk and Bell (1973), for contact arrest of cockroaches. In this case we see no evidence of consistent arrest for the majority of individuals, perhaps because the stimulus is uniformly distributed on the test substrate, rather than being localized. The *T. molitor* larvae wander over the surface of the papers in a random manner with the rate of activity decreasing through time (Weaver and McFarlane, 1989). As this rate of activity decreases, localized clusters of higher density form, particularly on DL-lactic acid-treated substrates but also, with time, on control substrates (Weaver and McFarlane, 1989). We suspect that yellow mealworm aggregations may involve mechanical interaction between individuals as well as other chemical messages. There can be little doubt that the overall response is acted out in concert with others behaving in the same manner. This complexity makes it difficult to view such chemicals as having a single effect. Their classification must depend, therefore, upon the relative effects upon both individuals and populations, not upon one or the other exclusively, as previously defined.

The response to lactic acid, a component of the frass only, may serve to indicate regions where infestations have been previously located. The long developmental time and conspicuous size of the larvae may make it advantageous for *T. molitor* to aggregate in these regions to avoid mechanical cleaning apparatus or cleaning procedures. This survival advantage may also be augmented by dispersion of the larvae when frass accumulates in amounts large enough to increase the lactic acid concentration into the repellent range. Such concentration increases could be spurred by further decomposition in the damp, dark regions *Tenebrio* inhabits. It is also of interest that acetic acid, which is present in both frass and food, induces no behavioral effects. This may be due to its environmental ubiquity precluding any directional advantage, but the lack

of response to a volatile major component of both food and frass is unexpected, given the fact that both are favorable materials for these larvae to inhabit.

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AGGREGATION IN YELLOW MEALWORMS, *Tenebrio molitor* L. (COLEOPTERA: TENEBRIONIDAE)

LARVAE

II. Observations and Analyses of Behavioral Parameters in Aggregation

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Abstract—Evaluation of the lactic acid attraction of individual and grouped larval *Tenebrio molitor* L. in an olfactometer indicated that olfaction is unlikely to be the chemoreceptive mode governing substrate choice or aggregation of these insects. High-magnification videotaped sequences of mealworms on treated and control filter papers indicated that larvae sample the substrate by rapidly probing with mouthpart palpi in a manner similar to the leaf sampling of certain caterpillars. The reception of lactic acid stimuli may therefore involve contact chemoreceptors. The larvae frequently touch each other in a similar manner. Bioassays comparing the cumulative frequencies of distributions of mealworms on control and lactic acid-treated filter papers indicated significant differences, with higher density clusters being found on the treated papers. Comparison of the control distribution with the expected distribution revealed an innate tendency to aggregate. The implications of these results are discussed with regard to the formation of mealworm clusters in the environment.

Key Words—Aggregation, lactic acid, olfactometer, mouthpart palpi, density, mealworm, *Tenebrio molitor*, Coleoptera, Tenebrionidae.

INTRODUCTION

Weaver et al. (1988) have shown that DL-lactic acid is an excreted aggregation pheromone found in larval *Tenebrio* frass. It may also act as an epideictic pheromone (Wynn-Edwards, 1962; Prokopy, 1981) if one considers the dispersal

ability of concentrations higher than those naturally occurring in the frass. McFarlane and Alli (1986) found lactic acid to be an excreted aggregant of *Blattella germanica*. L-Lactic acid is an olfactory host attractant for *Aedes aegypti* (Acree et al., 1968; Davis and Sokolove, 1976). Finger et al. (1965) found olfactory attraction was displayed between larvae of *Trogoderma granarium*, another stored products insect. The present study was undertaken to determine the chemoreceptive mode of yellow mealworms, with observations being conducted to determine whether olfaction or contact chemoreception was involved.

Experiments were also conducted to evaluate the density of distributions on lactic acid papers and on control papers using comparative bioassays. The possibility of multichemical stimuli being involved in mealworm aggregations is discussed.

METHODS AND MATERIALS

The larvae were reared as previously described (Weaver et al., 1989).

Olfactometric Analysis. A diagram of the olfactometer is shown in Figure 1. A Tygon T-type connector divided the purified airstream. Each branch was fitted with a 25-ml suction flask (H) to deliver test chemical stimuli (airflow rate 1.2 liter/min). The treatment flask contained 10 ml of test solution; the control flask contained 10 ml of deionized, glass-distilled water. In the case of dried test stimuli, six dried test strips (4 × 4 cm) were cut into 0.5-cm² pieces and placed in the test flask. Six dried distilled water test strips, prepared similarly, were placed in the control flask (flask positions were randomized). Beyond this, in each branch a 30-ml plastic vial was mounted horizontally (I) with a 0.5-cm airstream hole on the the stimulus delivery side and a 1.5-cm hole on the opposing side for insertion of the bioassay tube. These vials functioned as traps for insects committed to a given airstream. The traps were connected to opposing sides of a common starting arena (K) by 15 cm of 1.5-cm-diam. Tygon tubing (J) lined with fine mesh screening to facilitate larval movement. The starting arena vial was mounted vertically with two 1.5-cm-diam. holes opposing each other at the base. The cap of the arena vial was fitted with an airstream outlet to avoid subjecting future experimental animals to test odors. Individual larvae or 14 larval *T. molitor* were simultaneously introduced into the starting arena and their behavior was observed. Counts of mealworms in trap vials were taken at 2 and 4 hr. A solid CO₂ "smoke test" showed no evidence of airstream back-mixing. Ambient air temperature varied between 23°C and 25°C. The apparatus was disassembled after each usage and cleaned thoroughly using dilute acid, detergent, and serial distilled water rinses.

Videotaping Procedure. To determine how the larvae interact with each

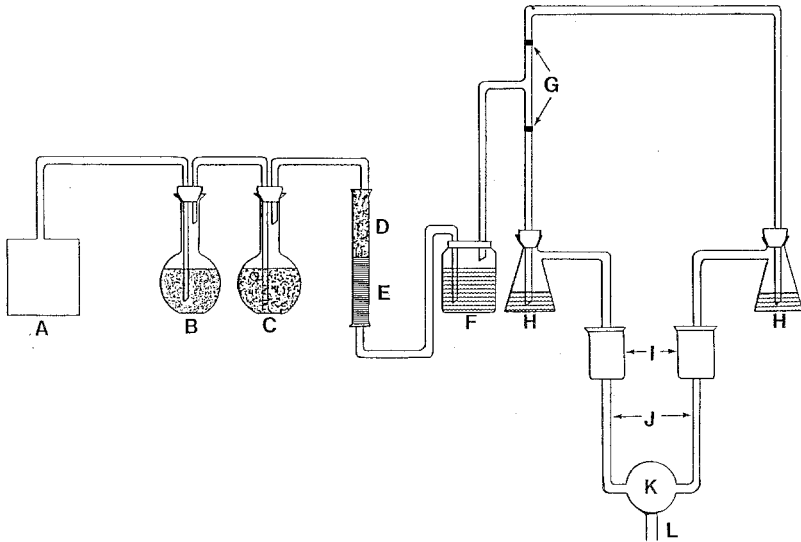


FIG. 1. Schematic diagram of olfactometer. A, vacuum pump coupled to rheostat; B, activated charcoal filter; C, drierite filter; D and E, gas purifier; F, gas washer; G, flowmeters; H, suction flasks (stimulus delivery); I, trap vials; J, Tygon crawl tubes; K, common starting arena; L, outlet to ventilation port.

other and the test substrate, the aggregation bioassay was set up as previously described (Weaver et al., 1989). Larvae were observed at 0.5-hr intervals and filmed through a Javelin JE 3010 color camera equipped with an EVA/Zoom lens (Javelin, Torrance, California). Taping was performed on a General Electric ICV 5025 videocassette recorder (Canadian General Electric, Montreal, Canada) at SP mode. Individual insects were observed for 1 min, if wandering, and 2 min, if arrested. Tapes were played back at 0.5 and 0.25 speed (real time) on a Hitachi CM-1481 monitor to quantify complex motions, particularly during wandering.

Bioassays for Comparing Population Distributions. A 5×45.5 -cm Whatman No. 1 filter paper strip was treated along its length with 3.5 ml of 5×10^{-3} M lactic acid and fixed into a cylindrical shape using a paper clip. This cylinder was introduced into a 19.2-cm-diam. glass specimen dish. The paper had been previously marked into nine equal 5×5 -cm grids using a carbon pencil. The cylinder was allowed to dry for 2 hr and then 30 mealworms were simultaneously introduced into the center of the cylinder. Counts of the number of larvae per grid were taken at 2, 4, and 6 hr; eight replicates were conducted, with arena positions randomized. Eight replicates of a similarly prepared distilled water control were conducted.

Statistical Analysis. The olfactometer data were subjected to the *t* test procedure, as were comparisons between mean numbers of insects/grid on treated and control papers in the population distribution bioassay (SAS Institute, 1982). Treated versus control population distributions were compared using the Kolmogorov-Smirnov two-sample test; control versus expected Poisson distributions were analyzed using the Kolmogorov-Smirnov one-sample test (Sokal and Rohlf, 1981).

RESULTS

The olfactometer bioassay indicates that larval *Tenebrio molitor* are unlikely to respond to airborne lactic acid molecules. Table 1 shows that yellow mealworms respond to both dried papers and solution-evolved stimuli in a manner not significantly different from the response to the control. This is true for both individual insects and groups of larvae. Table 2 illustrates the types of behavior displayed by *Tenebrio* larvae in the olfactometer bioassay. These data indicate that these larvae do show positive anemotaxis, with a total of 84.8% of the tested larvae reaching the trap vials. It is unlikely, therefore, that any behavioral bias against olfactometric analysis was overlooked.

Videotaping the larvae under high magnification revealed two predominant responses to the test chemical. Initially, mealworms wander over the filter paper surface with the following motion of the principal receptor organs: (1) antennae are rotated arbitrarily in all directions, making occasional contact with the papers; (2) maxillary palpi in a synchronous coordinated tapping activity which

TABLE 1. RESULTS OF LACTIC ACID OLFACTOMETER BIOASSAY

Chemical	Treated ^{a,b}	Control
Lactic acid (dry)		
10 ⁻¹ M	6.64 ± 0.50 ^b	5.79 ± 0.75 NS ^d
10 ⁻² M	4.86 ± 0.63 ^b	4.21 ± 0.81 NS
10 ⁻³ M	5.14 ± 0.69 ^b	4.86 ± 0.38 NS
10 ⁻⁴ M	5.71 ± 0.63 ^b	5.36 ± 0.75 NS
Lactic acid (wet)		
10 ⁻¹ M	5.50 ± 1.13 ^b	6.14 ± 0.44 NS
10 ⁻² M	6.50 ± 0.17 ^b	7.14 ± 0.69 NS
10 ⁻³ M	0.42 ± 0.06 ^c	0.54 ± 0.07 NS

^aMean number of larvae ± SEM

^bBased on eight replicates of 14 larvae each.

^cBased on 24 individual trials.

^dNS: Differences are not significant.

TABLE 2. TYPES OF BEHAVIOR OF GROUPED *Tenebrio* LARVAE^a IN OLFACTOMETER

Upwind crawling, reaching trap in ≤ 2 hr	63.4
Upwind crawling, reaching trap in ≤ 4 hr	9.8
Upwind crawling with ≥ 1 downwind shift; reaching trap in ≤ 4 hr	11.6
Upwind crawling with ≥ 1 downwind shift; not reaching trap in ≤ 4 hr	6.3
Quiescence, molting, or pupation.	8.9

^a $N = 112$ larvae.

generally contacts the paper two or three times per second, with occasional rates of up to five times per second; and (3) labial palpi are moved in either synchronous or asynchronous fashion, with contact occurring several times per second. Both maxillary and labial palpi contact the substrate for approximately 100 msec with a 100 to 500-msec lapse prior to the next contact. Occasionally, labial palpi may be moved in a similar fashion to the antennae with occasional paper contact rather than the above-described "probing." These occasional contacts occur when the head capsule is turned for a directional change. The other major response is that of larval arrest, in which the head capsule is curled forward so that antennae, maxillary, and labial palpi are generally all in constant contact with the test substrate. Aggregating larvae may not remain totally arrested but show occasional slow gregarious relocation. During wandering, the larvae generally display bursts of "probing" activity rather than doing so continuously. Larvae frequently come into contact with each other. This contact also involves much "probing" with palpi and antennae but is usually lacking the rapid synchronous maxillary tapping described above.

The results of the bioassay used to analyze distributions of *Tenebrio molitor* larvae are given in Figure 2. First, there were significant differences between control trials and those containing 5×10^{-3} M lactic acid test strips at each 2-hr interval. Secondly, these differences occurred at more than one larval density for each time interval. Differences become more pronounced with time. For example, at 2 hr, 100% (cumulative frequency = 1.0) of the larvae on the control were at grid densities of seven larvae per grid or less, whereas only 83.3% of the larvae on the treatment papers were found at this density or less. At 6 hr, 100% of the larvae on the control were located on grids containing seven yellow mealworms or less, whereas only 71.8% of the larvae on the treatment were found in grids containing ≤ 7 larvae. Finally, at both 4 and 6 hr, there are treatment clusters up to 13 or 14 larvae per grid, whereas on the control at 4 hr the highest density of larvae is eight per grid; at 6 hr the highest density on the control paper is six larvae per grid.

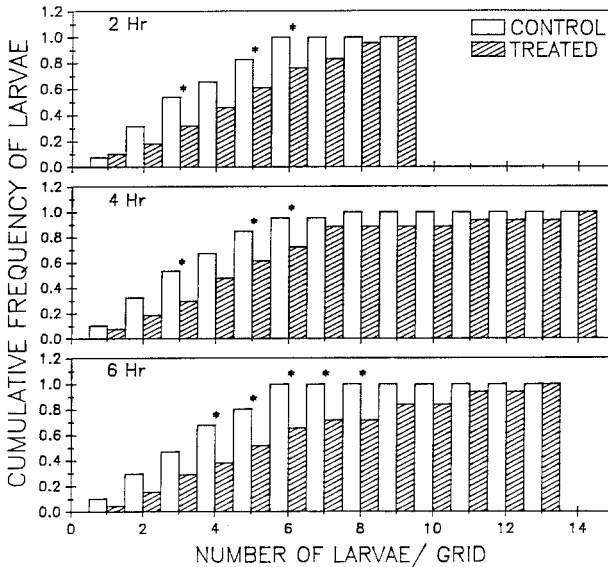


FIG. 2. Histogram comparing the cumulative frequency distributions of *Tenebrio molitor* larvae on control filter papers and those on filter papers treated with 5×10^{-3} M lactic acid at 2, 4, and 6 hr. Cumulative frequency at each abscissal point is the sum of all individuals at that density plus those at all preceding (lower) densities. *Cumulative frequency of treatment significantly less than control at $P \leq 0.001$. 2 hr: $n_1 = 174$, $N = 240$; $n_2 = 198$, $N = 240$; $D_{0.001} = 0.20257$. 4 hr: $n_1 = 172$, $N = 240$; $n_2 = 219$, $N = 240$; $D_{0.001} = 0.19862$. 6 hr: $n_1 = 155$, $N = 240$; $n_2 = 220$, $N = 240$; $D_{0.001} = 0.20767$.

There also is a tendency for a higher percentage of larvae to actually contact test substrates as opposed to control substrates, even when they are only presented one or the other. Two hundred forty insects were used in total in all treatment and also in all control trials. In the control trials, 174, 172, and 155 larval *Tenebrio* were found on the papers at 2, 4, and 6 hr, respectively. In the lactic acid trials, 198, 219, and 220 larvae were located on the papers at 2, 4, and 6 hr. The reduction in number of larvae at 6 hr in the control arenas is correlated with clusters that form at the base of the papers. These larvae (unstarved) defecate while on the papers. The fecal pellets accumulate to a substantial amount, and it is in these collections of frass droppings that the mealworms group.

Figure 3 displays the differences in mean numbers of larvae on 5×10^{-3} M lactic acid grids and control grids for occupied grids (excluding those without insects) and for all grids (including those that were empty).

A comparison of the control distribution with the expected Poisson fre-

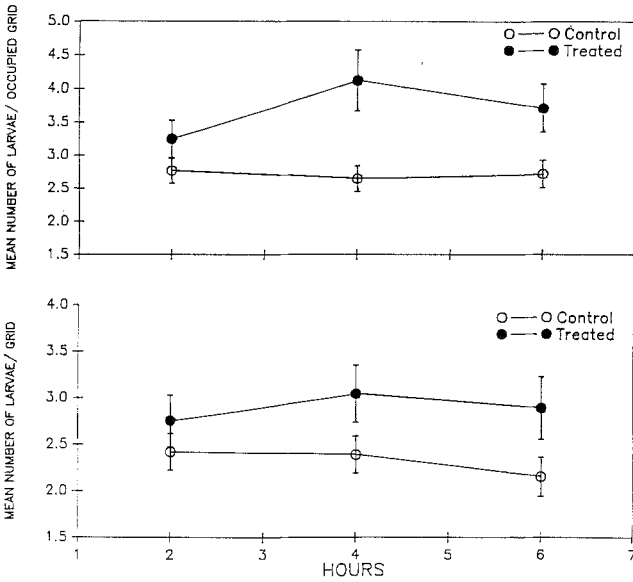


FIG. 3. Comparison of the mean number of larvae per occupied grid (top) and per all grids (bottom) on treatment or control papers at 2, 4, and 6 hr. For occupied grids treatment (5×10^{-3} M lactic acid) is significantly greater than control at $P \leq 0.001$ at 4 and 6 hr. For total grids treatment is significantly greater than control at $P \leq 0.01$ at 4 and 6 hr.

quency is shown in Figure 4. The frequency of larvae at lower densities is less than expected for certain values at the recorded time intervals. The cumulative frequency reaches or exceeds the expected value only for grids containing a higher number of larvae (at the highest density of 6 larvae/grid for 2 and 6 hr and at 8 larvae/grid at 4 hr). This indicates that the larvae tend to group in higher densities than theoretically expected. The cumulative frequency of larvae at lower densities also decreases with time on the control papers relative to the expected cumulative frequency. The larvae interact with each other in a similar manner, forming higher, as opposed to lower, density clusters, although with much lower density maximal clusters, than they do on treatment papers.

DISCUSSION

Olfactometric analyses indicate yellow mealworms do not respond to air-streams containing lactic acid in concentrations that successfully aggregate the larvae in the aggregation arena. Thus, olfaction is unlikely to be the chemoreceptive mode of *Tenebrio* larvae. This is to be expected, since the dispersion

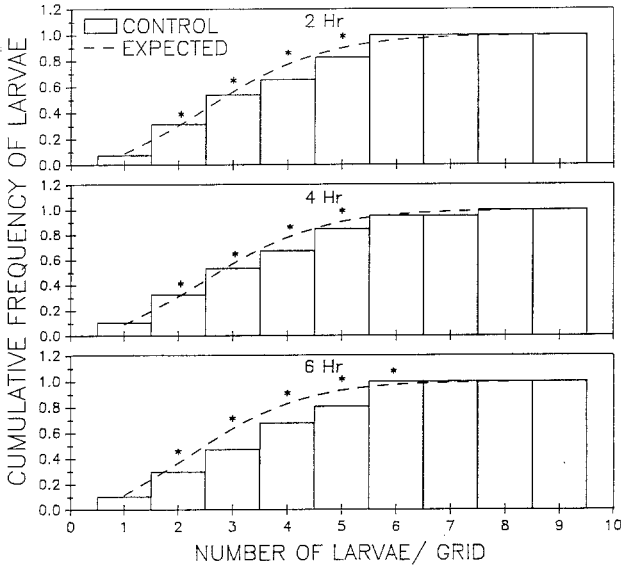


FIG. 4. Comparison of a histogram of the cumulative frequency distributions of *T. molitor* larvae on control filter with a plot of the cumulative frequency of the expected Poisson distribution at 2, 4, and 6 hr. Cumulative frequency at each abscissal point is the sum of all individuals at that density plus those at all preceding (lower) densities. *Cumulative frequency of the expected distribution (Poisson) of larvae significantly greater than the cumulative frequency of the larvae on control papers at the preceding larval density per grid; $P \leq 0.001$. 2 hr: $n = 174$, $D_{0.001} = 0.14779$. 4 hr: $n = 172$, $D_{0.001} = 0.14865$. 6 hr: $n = 155$, $D_{0.001} = 0.15659$.

of odor through the stored product mass would be uneven due to deflection away from unequal sized particles and collection in unevenly distributed air spaces. This would not provide a conventional concentration gradient for animal orientation. The lack of available water (Weaver et al., 1988) also would not provide for much aqueous evaporation to aid in the volatilization of the relatively low-volatility lactic acid.

The "probing" behavior viewed through videotaping suggests that mouthpart palpi are utilized to test the substrate for chemical information. The rapid maxillary tapping is similar to that described for larval *Choristoneura fumiferana* by Albert and Parisella (1988). This may be a behavior characteristic of feral *Tenebrio molitor* larvae (not those associated with stored products) that could be preadaptive for substrate sampling in stored foodstuffs. This does not suggest that purely mechanical stimuli are not also involved in such behavior.

After these animals arrive on the stimulus substrate they tend to wander rapidly across it, occasionally sampling it by "probing" in the predescribed

manner. This wandering brings the larvae into occasional contact. The individuals then tend to associate in a gregarious manner, with a conspicuous reduction in the rate of wandering, which may lead to group arrest. More often, however, individuals of these groups wander in a relatively random manner in an area in close association with the others; this wandering activity is much less rapid than that displayed after initial contact with the paper. This may involve an effect similar to that described by Burk and Bell (1973) for inhibition of cockroach locomotion by aggregation pheromone. Yellow mealworms may remain active because the bioassay does not provide localized regions of concentration. Frass accumulates to a very high density in *T. molitor* cultures, so stimuli can be continuously contacted by moving larvae.

These data suggest that: (1) mealworms show an innate tendency to aggregate, and (2) since they prefer lactic acid substrates, they might also prefer to aggregate (possibly in a different manner) on such substrates. The analysis of cumulative frequency distributions (Figures 2 and 4) indicates both are true. In addition, the movement of larvae into frass accumulated at the base of the control papers, but not from the treated papers, is convincing. The larvae on the treated paper may not do so because the lactic acid on the papers already delivers a "frass message." Both treatment and control responding larvae encounter this accumulated frass during slow wandering; only the control larvae show any pronounced preference for it.

These data allow for the suggestion of a possible scheme for the formation of mealworm clusters in these bioassays: (1) initially, larvae wander through the arena in a random manner and select a substrate for exploration; (2) next, they decide to remain upon this substrate or leave it after a period of rapid sampling; (3) if they leave, they may or may not return, via future random wandering, depending on the nature of the information further sampling provides (Weaver et al., 1989); (4) if groups of individuals select a substrate, they will encounter each other and tend to form loosely cohesive groups; (5) The chemical nature of the substrate may affect the density per unit area that individuals will tolerate, particularly if DL-lactic acid, a component of larval conspecific frass, is present. A second alternative would be that the presence of lactic acid initiates "conspecific-seeking" behavior after positively reinforcing the quality of substrate for these animals.

However, clusters are also formed on control strips in a similar manner, but at lower densities per grid. This innate tendency may also involve chemical messages related to the close proximity or contact between individuals. This may be similar to the chemotactile/mechanical aggregations among ovipositing locusts described by Norris (1970). This would mean that mealworm aggregation involves multichemical stimuli, with the unknown factor being a possible conspecific "recognition" or "interaction" factor. We are currently investigating this possibility.

It may be that the tendency for yellow mealworms to aggregate is related to an attraction to DL-lactic acid as an indicator of decomposing organic material suitable for these darkling beetles to feed upon in their feral state. The recent tendency of man to store harvested material may have provided abundant foodstuffs for an existing behavior to lead to enhanced survival by inhabiting those cryptic locations where deteriorating foodstuffs and frass may abound. Atkins (1966) states it is beneficial for aggregation to be selected for in cases where a resource is located at low frequency or sparsely distributed.

Alternatively, a mechanism may have evolved during the last 5000–20,000 generations (*T. molitor* development time 3–12 months, 5000 years of postharvest storage) whereby massive selection pressure occurred for attraction to a frass “marker.” Those individuals present in the main stored-products mass contributed little to the gene pool if they were constantly removed along with the food material or by cleaning procedures, whereas those present in cryptic locations remained to breed in these areas where fecal material, exuviae, and decomposing foodstuffs comprise nearly the entirety of the surrounding environment.

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ALLELOCHEMICALS IN TALL FESCUE—ABSCISIC AND PHENOLIC ACIDS

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Abstract—Growth inhibitors that can be leached from excised leaves of tall fescue grass (*Festuca arundinacea*) were investigated as allelochemicals. Leachates of desiccated Rebel and Kentucky 31 grass cultivars contained three principal inhibitory compounds, abscisic acid (ABA), caffeic acid, and *p*-coumaric acid. After quantitative analysis, abscisic acid was determined to be the predominant inhibitor. A 10-fold increase in ABA levels in leachates occurred after one day of desiccation. The concentration of ABA was 40% greater in Kentucky 31 leachate than in Rebel. This difference was also found in subsequent analyses of leachates of grasses that had been allowed to dry up to 30 days; however, the ABA concentration was reduced by 60% from the 10-fold increased levels.

Key Words—*Festuca arundinacea*, Gramineae, tall fescue, growth inhibition, allelopathy, abscisic acid, caffeic acid, *p*-coumaric acid.

INTRODUCTION

Several instances of apparent allelopathic interactions involving tall fescue have been described in the literature. Fields of tall fescue (*Festuca arundinacea* Schreb) were observed by Peters and Lun (1985) to have no other plant species growing in proximity, and this was considered to be the result of allelopathy. Mixed plantings of Kentucky 31 tall fescue and sweetgum [*Liquidambar styraciflua* (L.)] resulted in a diminution of growth of the sweetgum, which was attributed to interference (competition and allelopathy) by the fescue (Walters and Gilmore, 1976). An apparent allelopathic effect of tall fescue cultivars was

also found where differences in large crabgrass [*Digitaria sanguinalis* (L.) Scop.] infestation could be related to the cultivar of fescue previously grown in the soil. Aqueous leachates of tall fescue leaves and roots were shown to inhibit seedling growth of rape (*Brassica nigra* L.), birdsfoot trefoil (*Lotus corniculatus* L.), and red clover (*Trifolium pratense* L. (Peters and Lun, 1985).

Fractionation of these leachates into anionic, cationic, and neutral fractions was followed by bioassays. Inhibition of growth was found mainly with the anionic fraction. A number of organic acids present in this fraction, including lactic, succinic, malic, citric, shikimic, glyceric, fumaric, quinic, and some unknowns, were identified as inhibitory at a combined concentration of 37.5 mM. No mention was made of the presence of phenolic acids, which have been identified as a major group of allelopathic compounds present in similar concentrations in numerous other plants (Rice, 1984; Tang and Young, 1982). This study was begun to investigate whether tall fescue might be allelopathic when present in a mixture of turf grasses since many turf grass seed mixtures contain one or more cultivars of tall fescue. In this study, phenolic inhibitors were found to be present in leachates from tall fescue, and their level was high enough to cause growth inhibition. The major growth inhibitor, however, was found to be abscisic acid (ABA). In addition, an attempt was made to assess the importance of these compounds as growth inhibitory substances involved in this allelopathic system.

METHODS AND MATERIALS

Leaching. Rebel and Kentucky 31 tall fescue were grown in a greenhouse for 30 days in June and September, 1986, and leaves, after excision, were dried in the same location. Leachates were prepared by immersion of 150 g of fresh grass, or its equivalent after desiccation, in 1 liter of H₂O at 5°C for 24 hr.

Bioassay. The seedling growth assays were done using seeds of lettuce (*Lactuca sativa* Grand Rapids), annual ryegrass (*Lolium multiflorum* Lam.), creeping red fescue (*Festuca rubra* L., Ensylva), Kentucky bluegrass (*Poa pratensis* L., Kenblue), perennial ryegrass (*Lolium perenne* L., Manhattan), and Rebel tall fescue. The preliminary lettuce seed assay was performed in Petri dishes using 10 seeds and 1 ml of test solution with 3 replicates in a growth chamber set at 14 hr day and 10 hr night at 22 ± 1°C. The grass seed assays were done using 10 randomized replicates of 10 seeds with 1 ml of test leachate solution. The concentrations of leachate used were full strength and 1, 10, and 50% dilutions. Leaf and root lengths were measured after 7–14 days depending upon the grass species.

Isolation. The fescue leachate was adjusted to pH 8 and extracted with EtOAc. After adjustment to pH 3, the leachate was extracted twice with EtOAc,

resulting in an organic acid fraction. The original EtOAc fraction was extracted two times with 1 N HCl. The EtOAc layer was considered the neutral fraction. The acidic aqueous layer was made basic (pH 9) and extracted two times with EtOAc giving the basic fraction. The organic acid fraction was further fractionated for subsequent bioassay by HPLC using a semi-prep PRP-1 10 μm polystyrene 10 \times 250-mm column and solvent gradient system of MeCN and 0.006 N HCl (Buta and Lusby, 1986). Fractions found to be inhibitory were methylated with diazomethane in ether and analyzed by GC-MS. The GC-MS analysis was done on a HP5992A instrument equipped with a J&W DB-1701 15-m capillary column, 0.32 mm ID with 0.25 μm film thickness. The GC-EC analysis of ABA was done on a HP 5840A instrument equipped with an EC detector using a J&W SE54 30-m capillary column, 0.32 mm ID with 0.25 μm film thickness. The phenolic acids were quantified by HPLC with a UV detector at 280 nm and comparison with standard compounds. The quantitative determination of ABA in leachates was done by GC-MS isotope dilution analysis using an internal standard, the hexadeuterated analog of ABA (Rivier et al., 1977). At the start of the leaching period, 200 ng of the d_6 -ABA was added to the H_2O . The organic acid fraction was further separated by HPLC, and fractions containing ABA based on retention times were methylated with diazomethane and analyzed by GC-MS as above, except using selected ion current monitoring. Ions at m/z 190 and 194, corresponding to the base peak of the natural ABA and of its deuterated analog, were monitored. Dwell time was 50 msec for each ion.

Statistical Analysis The results of the grass seedling inhibition assays were analyzed by Dr. Marla McIntosh of the Statistical Consulting Group, USDA-ARS, Beltsville, Maryland. Fisher's LSD test for differences between treatment means was done.

RESULTS AND DISCUSSION

Excised leaves of Rebel and Kentucky 31 tall fescue grass grown in the greenhouse did not contain sufficient inhibitory substances to be detected in a lettuce seedling bioassay of the aqueous leachate. The only activity found was a stimulation of growth when the leachate was used at concentrations high enough that the levels of inorganic salts, potassium (19 $\mu\text{g}/\text{ml}$) and phosphorus (3 $\mu\text{g}/\text{ml}$), are comparable to those found in nutrient solutions (Buta et al., 1987). When the excised leaves of these two grasses were allowed to dry in air in the greenhouse for 30 days, high levels of inhibitory activity on the seedling growth of five turf grasses were found in aqueous leachates of both cultivars of tall fescue. These five turf grasses, which often occur in mixed plantings, were annual ryegrass, creeping red fescue *Ensylva*, Kentucky bluegrass *Kenblue*,

TABLE 1. EFFECT OF TALL FESCUE LEACHATES ON GRASS SEEDLING GROWTH

Leachate	Conc. (%)	Percent of control ^a				
		Annual Rye	Ensylva	Kenblue	Manhattan	Rebel
Grass leaf length						
Kentucky 31	1	111a	112a	112a	103ab	116a
	10	108ab	110a	108a	75c	105a
	50	71c	110a	99a	43d	68cd
	100	44d	85b	71b	31de	53de
Rebel	1	102ab	114a	113a	112a	86b
	10	95b	119a	95a	88bc	77bc
	50	76c	86b	57b	42d	38e
	100	25e	67c	36c	16e	20f
Grass root length						
Kentucky 31	1	112a	108b	127a	109a	108a
	10	91b	102b	90b	61b	74c
	50	28d	69c	56cd	34c	26e
	100	12e	41d	9e	16d	14ef
Rebel	1	115a	121a	114a	117a	91b
	10	59b	102b	66c	52b	57d
	50	39d	64c	41d	24cd	21ef
	100	13e	41d	20e	17d	12f

^a Means within a variety with the same letter are not significantly different. Fisher's LSD 0.05. Percent concentration of leachate derived from 150 g leaves/liter H₂O after 24 hr.

perennial ryegrass Manhattan, and tall fescue Rebel. Different levels of seedling growth inhibition or stimulation of the five grasses were found to be dependent on the concentrations of the leachates of the two fescue grasses tested (Table 1). The root and leaf growth of the grass seedlings was inhibited by high concentrations of two fescue leachates and stimulated by low concentrations. No major differences in inhibitory activity of the two fescue leachates were found on root lengths of the five grasses; however, the Rebel leachate was somewhat more inhibitory in its effect on leaf lengths. No effect on grass seed germination was found for treatments with the two leachates.

A partitioning of the leachate of Rebel was done to separate the components into acidic, basic, and neutral fractions. Inhibitory activity in the lettuce seedling bioassay was found in the organic acid fraction. This acidic fraction was chromatographed by reverse-phase HPLC, and three subfractions were found to contain inhibitory compounds, which reduced root growth to 50% of the control. The first inhibitory fraction contained a compound (No. 1) identi-

TABLE 2. INHIBITORY COMPOUNDS IDENTIFIED FROM LEACHATE OF REBEL TALL FESCUE

Compound No.	<i>m/e</i>	Compounds identified after methylation ^a	Original compounds
1	191, 207, 222 (M ⁺)	Methyl dimethoxycinnamate	caffeic acid
2	133, 161, 192 (M ⁺)	Methyl methoxycinnamate	<i>p</i> -coumaric acid
3	162, 190, 222	Methyl abscisate	abscisic acid

^aIsolated compounds were methylated by diazomethane.

fied as 3,4-dihydroxycinnamic (caffeic) acid, and the second inhibitory fraction contained a compound (No. 2) identified as 4-hydroxycinnamic (*p*-coumaric) acid, by UV and GC-MS. The third inhibitory fraction contained a compound (No. 3) causing both root and hypocotyl growth inhibition, and this compound was identified as the plant hormone abscisic acid (ABA) by GC-EC and GC-MS (Table 2). The same three compounds were also found in the inhibitory fractions of the partitioned Kentucky 31 leachates.

The concentrations of caffeic acid in the Rebel and Kentucky 31 grass (150 g/liter) leachates were 9 and 11 μ M, while those of *p*-coumaric acid were 14 and 23 μ M. These findings prompted an investigation of the concentrations of phenolic acids that were leached from fresh grass cuttings and grass dried for one day. The concentration of caffeic acid in both leachates for the two cultivars was less than 1 μ M. The concentration of *p*-coumaric acid was less than 1 μ M in the fresh grass leachate for both cultivars. The Rebel leachate of one-day-dried grass contained 1 μ M *p*-coumaric and the Kentucky 31 leachate contained 5 μ M. These concentrations, alone or in combination, were not sufficiently large to cause inhibition of seedling growth in a bioassay using Rebel seeds instead of lettuce seeds.

Since abscisic acid is involved in plant growth inhibition, senescence, and abscission (Takahashi, 1986), nanogram quantities of ABA found in the dried grass leachates by GC-EC analysis were sufficiently large, based on the bioassay results, to warrant further investigation. Experiments were designed to examine the time course of appearance of ABA after the leaves were excised and after they were allowed to dry in a greenhouse for a period of 30 days. In the first experiment, samples were collected for the first two days, after one week, and at the end of the experiment (data not shown). In the second experiment, two additional days of collection were added, and the results of the analyses for the *cis* and *trans* isomers of ABA are presented (Figure 1). These agreed with the findings in the first experiment. Concentrations of the *cis* isomer of ABA that were readily leached in 24 hr from leaves of fresh grass cuttings

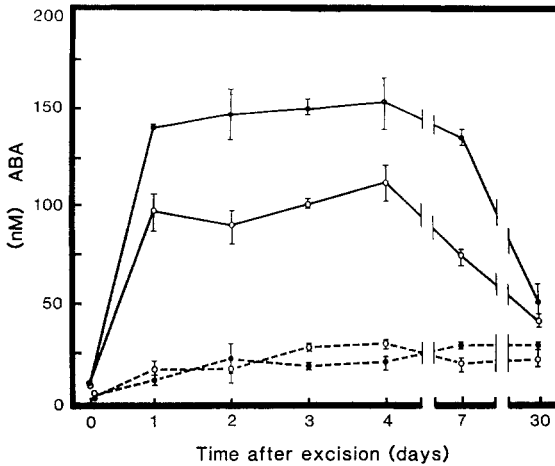


FIG. 1. Concentration of ABA in leachate from excised tall fescue leaves: *cis*-ABA (—) and *trans*-ABA (---) with cultivar Rebel (○) and Kentucky 31 (●); 150 g leaves/liter H₂O for 24 hr (means and ranges of duplicate samples).

were essentially the same for both Rebel and Kentucky 31. When 150 g of leaves were leached with 1 liter of water, the resulting concentration of the *cis* isomer was about 10 nM. The concentrations of *trans*-ABA found in the same leachate were 3.9 nM for Rebel and 2.8 nM for Kentucky 31. After one day of desiccation of grass (fresh weight 150 g), the concentrations of the *cis*-ABA found in the leachate of the desiccating grass increased to 97.9 nM for Rebel and 141.4 nM for Kentucky 31, while concentrations of *trans*-ABA were 15.7 nM for Rebel and 11.0 nM for Kentucky 31. Levels of both ABA isomers remained high in leachates obtained from grass dried for several days and diminished somewhat in the leachate of the grass dried for one week. After one month of desiccation, the leachate prepared from Rebel contained 42.8 nM of *cis*-ABA and 23.2 nM *trans*-ABA, while in Kentucky 31 leachate, 51.8 nM of *cis*-ABA and 28.5 nM of *trans*-ABA were found.

Observations have been made on the regrowth of frost-damaged grasses where exudates from these reduced the rate of plant growth, leaf elongation, and tiller production. These exudates inhibited seed germination, and this was not due to pH or osmotic potential. Similar effects were found by leaching dead grasses, but the levels of the inhibitory compounds were less. The bulk of the inhibition was thought to be due to tannins and phenolic compounds (Habeshaw, 1980).

The phenolic acids (*p*-coumaric and caffeic) found in the leachates of the dried tall fescue may not be as significant as ABA in turf grass growth inhibition

considering the concentrations of these compounds found in the leachates from the grasses desiccated for 30 days. Approximately 1 mM concentrations of phenolic acids, such as *p*-coumaric, were found to be necessary to retard barley root growth (Lynch, 1980), and this concentration is approximately 100 times the phenolic acid concentration found in the 30-day desiccated fescue grass leachates. Inhibition of radicle growth of cotton and wheat seedlings by mM concentrations of phenolic acids such as *p*-coumaric in bioassays were also reported in a study of the allelopathic effects of wheat mulch (Lodhi et al., 1987). Aqueous extracts of the ground mulch containing a number of phenolic acids, including *p*-coumaric, functioned as allelochemicals in inhibiting wheat and cotton seedling growth. Similar inhibitory activity was found with extracts of soil from a wheat field. The quantity of mulch used was three to five times greater than the quantity of dried fescue grass used in preparation of the leachates described in Methods and Materials. The concentration of phenolic acids found in the fescue leachates is certainly less than in the wheat mulch leachates described. Another study examining wheat straw leachates using other analytical methods concluded that the concentrations of phenolic acids were insufficient to cause the growth inhibition observed (Hall et al., 1986). Analysis of *p*-coumaric acid levels in the soil beneath California chapparal (*Adenostema fasciculatum*) indicated that there were 2 $\mu\text{g/g}$ of soil, or less than 1% of the quantity of this acid present in leaves on the plant, and it was concluded that the amounts of phenolic acids in the soil seemed to be too small to explain allelopathic effects (Proksch et al., 1985). Similar microgram per gram soil quantities of phenolic acids were found in soil containing decomposed ryegrass residue (Chou and Patrick, 1976).

The concentrations of ABA found in the desiccated tall fescue grass leachates are in the same concentration range as those from straw leachates [100 nM derived from 100-g samples of plant material leached with 1 liter of H_2O (Hall et al., 1986)]. The 100–140 nM ABA found in leachates from fescue after one day of desiccation are sufficiently high to be phytotoxic to barley roots within a few hours of application (Lynch, 1980). The approximately 100 nM concentrations of ABA found in fescue leachates have been found to be among the lowest concentrations of ABA causing root growth inhibition of maize (Pilet and Chanson, 1981). More recent studies have shown an inhibitory effect of exogenous 10 nM ABA on maize root elongation of a portion of a plant population (Pilet and Saugy, 1987). The concentrations of ABA resulting from leaching of desiccated fescue plant material in the field would be higher than the concentrations produced through the leaching process described in Methods and Materials, where sufficient water was used to completely cover the grass leaves. The likelihood that growth-regulating quantities of abscisic acid would be leached out of senescent or dehydrated plant parts, such as leaves, appears reasonable based on the quantities of ABA found upon leaching after the

extended desiccation of 30 days. Recent studies of wheat straw residues have shown nanomolar ABA concentrations in leachates of straw that had been left on the soil surface. When straw was incorporated into the soil after harvest, no ABA was detected nine weeks later (Hall et al., 1986). A similar fate for ABA leached from the tall fescue leaves would be expected.

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INFLUENCE OF (Z)-11-HEXADECEN-1-OL ON FIELD PERFORMANCE OF *Heliothis virescens* PHEROMONE IN A PVC DISPENSER AS EVIDENCED BY TRAP CAPTURE¹

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Abstract—Field studies were conducted to determine the effect of (Z)-11-hexadecen-1-ol (Z11-16:OH) on capture of tobacco budworm (TBW), *Heliothis virescens* (F.), males when added to two-component (HV-2) and six-component (HV-6) pheromone blends dispensed from a polyvinyl chloride (PVC) substrate. Numbers of males caught in traps baited with PVC dispensers containing 0.25% Z11-16:OH, relative to total pheromone content, were significantly higher than numbers caught with dispensers containing no Z11-16:OH. Catches of TBW were also increased significantly in some tests when Z11-16:OH was formulated in the bait at a 1% concentration. Catches were significantly reduced with dispensers containing 5.95% Z11-16:OH compared with those containing no Z11-16:OH. There were no differences in catch between traps baited with HV-2 vs. HV-6 or HV-2 + 0.25% Z11-16:OH vs. HV-6 + 0.25% Z11-16:OH when formulated in PVC substrate.

Key Words—Tobacco budworm, *Heliothis virescens*, Lepidoptera, Noctuidae, polyvinyl chloride, pheromone dispensers, pheromone blends, (Z)-11-hexadecen-1-ol.

¹Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

INTRODUCTION

Two components of the tobacco budworm (TBW), *Heliothis virescens* (F.), sex pheromone complex were identified in independent studies by Roelofs et al. (1974) and by Tumlinson et al. (1975) and were reported by these two groups to be (Z)-11-hexadecenal (Z11-16:Al) and (Z)-9-tetradecenal (Z9-14:Al) in the approximate ratio of 16:1. This blend of the two chemicals was attractive to males, although Tumlinson et al. (1975) speculated that the actual pheromone blend also contained other components based on behavioral data. Klun et al. (1980) subsequently identified five additional components from pheromone gland rinses including (Z)-9-hexadecenal (Z9-16:Al), (Z)-7-hexadecenal (Z7-16:Al), (Z)-11-hexadecen-1-ol (Z11-16:OH), hexadecanal (16:Al), and tetradecanal (14:Al). Some reports concerning the relative biological effectiveness of various blends of the components seem to conflict. Blends previously tested by several workers have contained only Z11-16:Al and Z9-14:Al, while other blends contained the full complement of seven components. Generally, however, these tests have been performed with blends formulated in different substrates. For example, Sparks et al. (1979) reported that the seven-component blend was significantly better in field trapping studies than the two-component blend when dispensed from newly prepared cotton wick dispensers that were placed in traps each night.

Hartstack et al. (1980) observed no significant differences in captures of male TBW in traps baited with either the two-component or the seven-component blends. Both blends were formulated in rubber septa and laminated plastic dispensers. Hendricks (1982) also found that the two-component blend and seven-component blend caught about equal numbers of moths when each mixture was dispensed from polyvinyl chloride (PVC) capsules. Hendricks et al. (1987) showed that the two-component blend incorporated into black molded PVC performed as well as a six-component blend (Z11-16:OH omitted) formulated in the PVC. Ramaswamy et al. (1985) reported that a four-component blend of Z11-16:Al, Z9-14:Al, 16:Al, and Z11-16:OH at a ratio of 65.1:1.6:32.6:0.7 dispensed from rubber septa was an optimal sex pheromone blend for attracting male TBW and that blends containing no Z11-16:OH or those containing more than 1% Z11-16:OH in relation to Z11-16:Al were generally found to be suboptimal.

Tumlinson et al. (1982), Pope et al. (1982), and Teal et al. (1986) found no Z11-16:OH in the volatile pheromone components released from the pheromone gland or in volatiles trapped from actively calling females. Vetter and Baker (1983) evaluated blends of pheromone components with and without Z11-16:OH in studies utilizing a flight tunnel and could find no evidence that the alcohol was involved in behavioral functions under those conditions.

The purpose of our experiments was to determine if addition of Z11-16:OH

to various blends of the six other pheromone components, dispensed from PVC, would affect trap catch of TBW males.

METHODS AND MATERIALS

Pheromone Dispensers. Liquid vinyl chloride (VC) homopolymer (black, S-51-F89) was supplied by Sinclair & Rush, Inc. Pheromone components (95.7–98.5% purity) were supplied by Zoecon Corp., Palo Alto, California; Chem Samp Co., Columbus, Ohio; and Shin Etsu, Japan. Purity was verified by capillary gas chromatography on 50-m OV-1701 and 50-m SP-2330 columns. Liquid VC-pheromone mixtures were molded according to the procedure of Hendricks et al. (1987) to give molded polyvinyl chloride (PVC) dispensers. Individual dispensers were cut from both rods and sheets to give pieces that weighed 0.65 ± 0.2 g and a pheromone dosage per bait of approximately 6 mg active ingredient (AI). Table 1 shows the relative proportions of chemicals in each formulation tested.

Field Test. Inverted 75–50 cone traps (Hartstack et al. 1979) were used in all bioassays conducted in these experiments. These traps were installed so that the access hole (cone bottom) was at a height near the top of surrounding host plants. Host plants were irrigated cotton in Burleson County, Texas, and either irrigated cotton or wild sunflowers in Cameron County, Texas. Traps were positioned at least 20 m apart in tests conducted in Cameron County and at least 50 m apart in tests conducted in Burleson County. The test pheromone dispensers (baits) were suspended 3 cm below the trap at the center of the open cone.

Field tests were conducted to determine the effect of adding varying amounts of Z11–16:OH to a blend of HV-2 or of HV-6 (Table 1). Z11–16:OH

TABLE 1. PERCENTAGES OF CHEMICALS BLENDED IN 2-COMPONENT AND 6-COMPONENT MIXTURES OF *H. virescens* SEX PHEROMONE IN PVC SUBSTRATE

Chemical	Percentage (proportion)/ formulation	
	HV-2	HV-6
(Z)-11-Hexadecenal (Z11–16:AI)	93.7 (14.8)	78.4 (14.8)
(Z)-9-Tetradecenal (Z9–14:AI)	6.3 (1.0)	5.3 (1.0)
(Z)-9-Hexadecenal (Z9–16:AI)		1.8 (0.3)
(Z)-7-Hexadecenal (Z7–16:AI)		1.8 (0.3)
Hexadecanal (16:AI)		9.2 (1.7)
Tetradecanal (14:AI)		3.5 (0.7)

^aProportions were calculated using the amount of Z9–14:AI as (1.0) part.

was added to the blends at concentrations of 0.25%, 1.0%, and 5.95% of the total pheromone content of the individual dispensers. In one test, HV-2 and HV-2 + Z11-16:OH formulated in PVC were compared with a blend of Z11-16:Al (1000 μg), 16:Al (500 μg), Z9-14:Al (25 μg) and Z11-16:OH (10 μg) formulated in rubber septa according to the method of Ramaswamy et al. (1985). Since previous work has indicated that this formulation is very effective in trapping TBW males during the first four nights of use but that efficacy falls off rapidly on subsequent nights (Lopez et al., 1987); rubber septa containing pheromone were replaced every four days. PVC lures were not replaced during the 16-day duration of the test. All other tests were conducted for 10- to 14-day periods with five replicates per dispenser category. The position of each dispenser was initially randomized within each replicate, and then positions were rotated within the replicate daily to reduce bias caused by trap location. Traps were set in lines perpendicular to prevailing winds (SE).

Data were analyzed by a nonparametric *t* test (Ostle, 1969).

RESULTS AND DISCUSSION

Field experiments in which traps were baited with molded PVC dispensers containing Z11-16:Al and Z9-14:Al (14.8:1 ratio) plus varying quantities of Z11-16:OH indicated that small quantities of Z11-16:OH enhanced the performance of the dispenser as indicated by trap catch (Table 2). In tests in Burle-

TABLE 2. COMPARISON OF NUMBERS OF *H. virescens* MALES CAUGHT IN TRAPS BAITED WITH HV-2 CONTAINING VARYING AMOUNTS OF Z11-6:OH FORMULATED IN PVC RODS, 6 mg/lure, BURLESON AND CAMERON COUNTIES, TEXAS, 1984

Formulation ^a	Mean no. of <i>H. virescens</i> males caught/trap/night ^b		
	1984	1986	
	Burleson County	Burleson County	Cameron County
HV-2 + 0.25% Z11-16:OH	32.3 a	48.0 a	21.0 a
HV-2 + 1.0% Z11-16:OH	35.9 a	40.4 ab	9.4 b
HV-2	21.3 b	38.1 b	7.6 b
HV-6	— ^c	36.1 b	9.2 b
HV-2 + 5.95% Z11-16:OH	13.2 c	16.5 c	4.5 c

^aHV-2 and HV-6 formulations are described in Table 1. Percentage Z11-16:OH was calculated on the basis of total pheromone content.

^bMeans in the same column followed by same letters were not significantly different ($P \leq 0.05$, $df = 54$, $t \leq 2.01$ for Burleson County, 1986; $P \leq 0.05$, $df = 82$, $t \leq 1.99$ Cameron County, 1986; $P \leq 0.05$, $df = 58$, $t \leq 2.0$ for Burleson County, 1984).

^cNot tested.

son County and Cameron County, Texas, in 1986, those lures containing the lowest quantity of Z11-16:OH (0.25% of the total pheromone content) caught significantly more male TBW moths than dispensers with no Z11-16:OH or those containing high levels of Z11-16:OH (5.95%). Also, traps baited with dispensers containing high levels of Z11-16:OH caught significantly fewer male TBW moths than those without Z11-16:OH. There were no significant differences between mean catches in traps baited with PVC dispensers containing HV-2 and HV-6. In a test in Burleson County in 1984, the same pattern was observed except that moth catches were greater in traps baited with dispensers containing HV-2 plus 1.0% Z11-16:OH than in traps baited with HV-2 alone. Dispensers containing 5.95% Z11-16:OH caught fewer moths than traps baited with dispensers containing no Z11-16:OH.

The effect of Z11-16:OH was tested in June, July, and August 1987, when captures of TBW in pheromone traps were low, medium, and high, respectively, to determine if the number of TBW being captured influenced the effect of Z11-16:OH on field performances of the pheromone in PVC dispensers containing HV-6. Traps baited with dispensers containing 0.25% and 1.0% Z11-16:OH caught more male TBW than traps baited with dispensers containing no Z11-16:OH at all three capture levels except in the July test, where the number caught by dispensers containing 1.0% Z11-16:OH was numerically greater. However, the difference was not significant at $P < 0.05$ (Table 3). Traps baited with dispensers containing 5.95% alcohol caught significantly fewer TBW moths than traps baited with dispensers containing no Z11-16:OH or 0.25% or 1.00% Z11-16:OH.

In 1986, a comparison was made using PVC formulations of HV-2 and HV-6 alone or with 0.25% Z11-16:OH. In this comparison, dispensers con-

TABLE 3. COMPARISON OF NUMBERS OF *H. virescens* MALES CAUGHT IN TRAPS BAITED WITH HV-6 CONTAINING VARYING AMOUNTS OF Z11-16:OH, FORMULATED IN PVC RODS, 6 mg/lure, BURLESON COUNTY, TEXAS, 1987

Formulation ^a	Mean no. of <i>H. virescens</i> males caught/trap/night ^b		
	June 1987	July 1987	August 1987
HV-6 + 0.25% Z11-16:OH	10.3 a	87.4 a	136.7 a
HV-6 + 1.0% Z11-16:OH	10.0 a	67.9 ab	131.6 a
HV-6	6.7 b	52.2 b	89.6 b
HV-6 + 5.95% Z11-16:OH	3.7 c	26.3 c	67.3 c

^aHV-6 formulation is described in Table 1. Percentage Z11-16:OH was calculated based on total pheromone content.

^bMeans followed by same letters were not significantly different, ($P \leq 0.05$, $df = 190$, $t \leq 1.97$ for June; $P \leq 0.05$, $df = 190$, $t \leq 1.97$ for July; $P \leq 0.05$, $df = 130$, $t \leq 1.98$ for August).

TABLE 4. COMPARISON OF NUMBERS OF *H. virescens* MALES CAUGHT IN TRAPS BAITED WITH HV-2 AND HV-6 ALONE AND WITH 0.25% Z11-16:OH IMPREGNATED IN PVC SHEETS, 6 mg/lure, BURLESON COUNTY, TEXAS, 1986

Formulation ^a	Mean no. of <i>H. virescens</i> males caught/trap/night ^b
HV-6 + 0.25% Z11-16:OH	88.6 a
HV-2 + 0.25% Z11-16:OH	88.5 a
HV-6	54.5 b
HV-2	48.1 b

^aHV-2 and HV-6 formulations are described in Table 1. Percentage Z11-16:OH was added at a rate of 0.25% of the total pheromone.

^bMeans followed by same letters were not significantly different ($P \leq 0.05$, $df = 138$, $t \leq 1.98$).

taining HV-2 and HV-6 plus 0.25% alcohol caught significantly more male *H. virescens* than dispensers containing HV-2 and HV-6 without alcohol (Table 4). No differences were observed in comparisons of HV-2 and HV-6 or between HV-2 + Z11-16:OH and HV-6 + Z11-16:OH.

In the test comparing the rubber septum formulation proposed by Ramaswamy et al. (1985) and the PVC formulations of HV-2 and HV-2 + 0.25% Z11-16:OH, the Ramaswamy dispenser replaced every fourth day caught significantly more male TBW moths than either of the HV-2 PVC formulations. Also, the HV-2 PVC formulation supplemented with 0.25% Z11-16:OH caught significantly more moths than the HV-2 PVC formulation containing no Z11-16:OH. However, the latter two dispenser types (PVC) were not replaced during the 16-day test period (Table 5).

TABLE 5. COMPARISON OF NUMBERS OF *H. virescens* MALES CAUGHT IN TRAPS BAITED WITH HV-2 AND HV-2 + Z11-16:OH FORMULATED IN PVC RODS OR 4-COMPONENT BLEND FORMULATED IN RUBBER SEPTA, BURLESON COUNTY, TEXAS, 1986

Formulation and substrate	Mean no. of <i>H. virescens</i> males caught/trap/night ^a
4-component blend, septa ^b	88.8a
HV-2 + 0.25% Z11-16:OH, PVC	68.7b
HV-2, PVC	39.6c

^aMeans followed by same letters were not significantly different ($P \leq 0.05$, $df = 82$, $t \leq 1.99$).

^bLure was replaced every four days; rubber septa was formulated according to Ramaswamy et al. (1985). PVC lures were not replaced during the 16-day test. Four-component blend was Z11-16:Al, Z9-14:Al, 16:Al, and Z11-16:OH at a ratio of 65.1:1.6:32.6:07.

These tests have demonstrated that the addition of 0.25 or 1.00% of Z11-16:OH to PVC formulations containing either HV-2 or HV-6 enhanced the performance of the dispensers in field trapping studies using inverted cone traps. However, addition of 5.95% of the alcohol to the formulation reduced the effectiveness of the lure. The 5.95% Z11-16:OH used in this study was equivalent to the proportion of Z11-16:OH used by Klun et al. (1980) in field bioassays of attraction of TBW males to mixtures of compounds identified from TBW females. Our results agree with the findings of Ramaswamy et al. (1985), who reported a similar response when pheromone was formulated in rubber septa.

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IDENTIFICATION OF THE SEX PHEROMONE OF THREE *Matsucoccus* PINE BAST SCALES

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Abstract—*Matsucoccus resinosae* in the United States, *M. matsumurae* in China, and *M. thunbergianae* in Korea use (2*E*, 4*E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one (**1**) (matsuone) as the primary component of their sex attractant pheromones. The structure was postulated from mass and NMR spectra and confirmed by synthesis of analogs **3**, (2*E*, 4*E*)-4,6,11,12-tetramethyl-2,4-tridecadien-7-one, and **4**, (2*E*, 4*Z*)-4,6,11,12-tetramethyl-2,4-tridecadien-7-one. Both analogs were attractive to the males of *M. resinosae* in laboratory bioassays and to *M. matsumurae* in laboratory and field tests, but the 4*Z* analog (**4**) was much less so than the 4*E* analog (**3**) and had inhibitory effects at high concentrations. Dodecanol, isolated from aeration and solvent extracts of female *M. resinosae*, evoked characteristic wing-raising by pedestrian males, but the role of this response was not determined.

Key Words—*Matsucoccus resinosae*, *M. thunbergianae*, *M. matsumurae*, sex pheromone, analog, dodecanol, Homoptera, Coccoidea, Margarodidae, (2*E*, 4*E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one, matsuone.

INTRODUCTION

Despite their great economic significance and diversity, research on semi-chemical-mediated sexual behavior of scale insects and mealybugs (Coccoidea) has been relatively recent and limited. The first report of a sex attractant

in the superfamily was for the red pine scale, *Matsucoccus resinosae* Bean & Godwin (Doane, 1966). Warthen et al. (1970) isolated the sex pheromone of the California red scale, *Aonidiella aurantii* (Maskell), and the chemical structures of the active compounds were described seven years later (Roelofs et al., 1977). To the present date, sex pheromone structures for three additional armored scales (Diaspididae) and two mealybugs (Pseudococcidae) have been reported. Three sex pheromone components have been identified from the San Jose scale, *Quadraspidiotus perniciosus* (Comstock), (Gieselmann et al., 1979b; Anderson et al., 1981), and two from the California red scale (Roelofs et al., 1977). Single component attractants were identified for the yellow scale, *Aonidiella citrina* (Coquillett), (Gieselmann et al., 1979a); the white peach scale, *Pseudaulascaspis pentagona* (Targioni-Tozzetti), (Heath et al., 1979); the Comstock mealybug, *Pseudococcus comstocki* (Kuwana), (Bierl-Leonhardt et al., 1980); and the citrus mealybug, *Planococcus citri* (Risso), (Bierl-Leonhardt et al., 1981). The multiple components of the San Jose scale and the California red scale are individually attractive and apparently not synergistic. The alcohol corresponding to the propanoate sex attractant of the white peach scale acts as a short-range sex stimulant but is not an attractant (Einhorn et al., 1983). Thus, for the purpose of bringing the sexes together, the multiple components are apparently redundant; effectively, all Coccoidea investigated to date respond to single components.

Analogues of California red scale pheromones and the closely related yellow scale were not active (Roelofs et al., 1977, 1978, 1982; Anderson and Henrick, 1979; Gieselmann et al., 1980) while pheromone analogues of the white peach scale (Heath et al., 1979), the citrus mealybug (Bierl-Leonhardt et al., 1981), and the Comstock mealybug (Mori and Ueda, 1981; Uchida et al., 1981; Bierl-Leonhardt et al., 1982) showed somewhat inferior activity.

Sex pheromones were structurally different (Roelofs et al., 1982) and not cross-attractive between *Aonidiella* species (Moreno et al., 1972) or *Planococcus* species (Tremblay and Rotundo, 1976). In contrast, cross-attraction was complete among *M. resinosae*, *M. matsumurae* (Kuwana)⁴ (Young et al., 1984), and *M. thunbergianae* Miller and Park (Park et al., 1986), partial between *M. matsumurae* and *M. massoniana* Young and Hu (Young and Qi, 1983), and slight between *M. massoniana* and *M. resinosae* (Young et al. 1984). Pheromones of *M. matsumurae* (Qi et al., 1983), *M. josephi* (Sternlicht et al., 1983), and *M. resinosae* (Park et al., 1986) have been isolated. We report the chemical structure of the primary component of the sex attractant pheromone isolated from *M. resinosae* in the United States, *M. matsumurae* in China, and *M. thunbergianae* in Korea. We also report identification of a compound from female

⁴McClure (1983) and Young et al. (1984) suggested that *M. resinosae* might be a junior synonym of *M. matsumurae*, but the names have yet to be formally proposed.

M. resinosa that evokes a wing-raising response, but not taxis, by males of that species. Lastly, we present a comparison of laboratory bioassays of male *M. resinosa* response to analogs of its pheromone and to a crude extract of pheromone from *M. thunbergiana* females.

METHODS AND MATERIALS

Chemistry of Pheromone and Analogs. In August 1982, 1984, and 1985, respectively, extracts of 3500, 17,000, and 26,500 female *M. resinosa* were fractionated as previously described (Park et al., 1986). A component that elicited attraction was in subfraction 3-5-4 and a component that evoked wing-raising was in fraction 2. A unit-resolution, EI mass spectrum (70 eV) was obtained for the attractant on a Finnigan 4000 instrument fitted with a DB-1, 30-m capillary column, and for the wing-raising component on a Finnigan 3000 fitted with a 1-m OV-101 packed column.

The tentative identification of the compound that elicited wing-raising was confirmed as dodecanol by coinjection with an authentic sample on a 6.1-m \times 3-mm-ID glass column, 6% Carbowax 20 M and on a 3-m \times 3-mm-ID glass column, 4% FFAP, and also by congruence of the mass spectrum with that of an authentic sample. A high-resolution mass spectrum on the sex attractant component was obtained on a Kratos MS 50TC spectrometer fitted with a 30-m DB-1 column. The same components were also identified in the volatiles obtained by aerating the female *M. resinosa* for five days prior to extracting them; purified air (charcoal) was passed over the insects on crumpled filter paper in a flask, and the volatiles were collected on Porapak Q, from which they were extracted with pentane.

An extract in 1 ml hexane of 400 female *M. thunbergiana* collected from infested *Pinus thunbergiana* in the southwestern extremity of Korea was used for cross-attraction bioassays against *M. resinosa* males (Park et al., 1986) and for coinjection with extracts of *M. resinosa* and *M. matsumurae* on a 30-m DB-1 capillary column programmed, after 40 sec at 35°C, to 200°C at 4°/min; a single sharp peak at 34.8 min represented the attractant component common to the three samples. *M. resinosa* and *M. thunbergiana* extracts were also coinjected on a 50-m FFAP capillary column under the same conditions.

In the spring of 1983, approximately 30,000 female *M. matsumurae* were collected from *Pinus tabulaeformis* in the suburbs of Wuxi, Jiangsu Province and in Haiyang County, Shandong Province. Purified air (molecular sieves) was passed over these insects for seven days, and the volatiles were trapped on Tenax (see Qi and Burkholder, 1982, for general procedure), which was then extracted with hexane. The active fraction was eluted from a silica gel column (35 \times 0.6 cm, 200–320 mesh) with 2% ether in hexane, and the active subfrac-

tion was eluted from a Florisil column impregnated with 15% silver nitrate (35×0.6 cm, 120–150 mesh) with 10% ether in hexane. This active subfraction was concentrated, and aliquots were repeatedly injected on a 40-m \times 0.3-mm-ID PEG capillary column at 158°C, N₂ 2 cc/min. The collected active peak (retention time 33 min) in 3.5 ml hexane gave a unit-resolution mass spectrum that was congruent with that obtained by GC-MS on subfraction 3-5-4 from *M. resinosa* (above). A GC-FTIR (vapor phase) was obtained on an IBM IR98 fitted with a DB-1 capillary. To obtain an NMR spectrum, the *M. matsumurae* sample was injected on a 1.5-m \times 9-mm-ID glass column, 3% OV-101 on Chromosorb G 50/80, 140°C, He 80 cc/min. The peak at 18–22 min was collected in a 25-cm \times 1.7-mm-OD glass capillary under thermal gradient (Brownlee and Silverstein, 1968) and rinsed with 0.4 ml 100% C₆D₆ into a 5-mm-OD NMR tube. The NMR spectrum was obtained on a General Electric GN 500 spectrometer.

Unit resolution mass spectra were obtained on two synthesized analogs (**3** and **4**) under the same conditions above for the primary attractant component. Compounds **3** and **4** were separated on a 2.4-m \times 6-mm-ID glass column, 4% TCEP on Chromosorb G 60/80, 140°C, N₂ 60 cc/min. 500-MHz NMR spectra on **3** and **4** were obtained under the same conditions used for the isolated primary sex attractant component.

Preparing Dilutions of Synthetic Pheromone Analogs and Crude Female Extract. Two pheromone analogs were synthesized: a positional isomer of the *M. resinosa* pheromone component, **3**, and its geometric isomer, **4**. These compounds were prepared at concentrations of 5 ng in 1 μ l hexane and 1 μ g in 1 μ l hexane, respectively. The original dilution of compound **3** was further serially diluted so that each 1 μ l hexane dilution contained 0.5 ng, 0.05 ng, 5 pg, and 0.5 pg; the original dilution of compound **4** was also serially diluted to 0.1 μ g, 0.01 μ g, 1 ng, 0.1 ng, and 0.01 ng in 1 μ l hexane.

A crude pheromone extract was made by immersing in 1 ml of hexane 200 newly emerged *M. thunbergiana* females collected from a laboratory colony at Seoul on March 18, 1986. After three days of storage at room temperature, the crude extract was serially diluted to 10%, 1%, 0.1%, and 0.01%. The amount of extracted pheromone in 1 μ l of each dilution was 2×10^{-2} , 2×10^{-3} , 2×10^{-4} , and 2×10^{-5} female equivalents (FE), respectively. The original crude extract and its hexane dilutions were stored at -10 ± 5 °C.

Preparation of Males for Testing. Branches of red pine (*Pinus resinosa*) containing cocoons of *M. resinosa* were collected from Dutchess County, New York, in September 1987 and put in emergence cages made of cardboard boxes with three Petri dishes attached at one side of each cage. The cages were stored at 20 ± 4 °C under natural day light. Because the males show strong positive phototaxis, newly emerging males could be collected from inside the Petri dishes.

Bioassays with Medicine Droppers. The bioassay procedures using medicine droppers were those of Park et al. (1986). Each test male was kept under a numbered, inverted, transparent plastic cup (ca. 20 ml) until tested. After each test, the male was covered with the same cup and kept for the next test. The stimulus was delivered to walking males, on a sheet of white paper, in puffs from a medicine dropper that had been charged with 1 μ l of solution placed with a micropipet about 1 cm inside the tip. The dropper tip was positioned to one side of a walking male, about 8 mm from the antennae. Puffs of air were then forced from the medicine dropper by gently pressing the bulb at 1.5-sec intervals. The attraction was measured as degree of following towards the retreating dropper tip. Males that followed for one or more sides of a 4-cm-sided equilateral triangle were given scores from 1 to 3, according to the number of triangle sides completed; males responding to the attractant but following less than one side were given a score of 0.5, and those who did not follow were given a score of 0.

On each day of this experiment, between five and eight males were used at 5-min intervals in successive tests of each dilution. A 1% dilution of the crude female extract (2×10^{-3} FE) was used as a standard. Only actively walking males that had a response score of 3 to the standard were used. Males that became sluggish were replaced as needed to complete the test series.

The bioassays were done at $22 \pm 1^\circ\text{C}$, $68 \pm 7\%$ relative humidity, and 500–700 lux of fluorescent light between 8:00 AM and 12:30 PM from September 6 to 27, 1987. The number of stimuli varied among the days. Bioassays always proceeded from the lowest concentration to the highest, and the order for the tests with dilutions of crude extract, **3**, and **4** was alternated each day. The data were analyzed in a completely randomized design and subjected to Duncan's multiple-range test.

Dummy Female Experiments. Dummy females were made by rolling a 4×10 -mm filter paper coated with rice paste to form a cylinder 4 mm long and 1.5 mm in diameter, similar in shape and size to a female. Each dummy female was treated with 1 μ l of test solution, and held for 2 min to allow the hexane to evaporate before the test. The dummy female was placed perpendicular to the path of the walking male, ca. 1 cm away, and the male behavior was observed. Bioassays were done at $22 \pm 1^\circ\text{C}$, $65 \pm 3\%$ relative humidity, and 700 lux of fluorescent light between 10 AM and 3 PM from September 25 to October 8, 1987. Males were selected and replaced as in the medicine dropper bioassays, and allowed a rest of about 10 min between tests.

Laboratory and Field Bioassays of Compounds 3 and 4 Using M. matsurae in China. The laboratory bioassay used was the Petri dish method described by Qi et al. (1983). The assay was conducted by placing two 1-cm pieces of filter paper, one containing the material to be tested, the other, an appropriate solvent blank, into a 7-cm-diameter Petri dish. After the solvent

had evaporated, 10 males were admitted and the Petri dish was immediately covered. After a 2-min adaptation period, the number of males on the sample disk versus the number of males on the blank disk was counted every 2 min over a period of 20 min.

The field experiments were conducted in the suburbs of Wuxi, PRC, from April 26 to 29, 1988. The trap consisted of a piece of cardboard (22 × 26 cm) that was folded so that the 26-cm side was bisected; the angle of the fold was 60°. Both sides of the trap were coated with Vaseline and a hole was cut (1 cm) in each flap. For these tests, a rolled piece of filter paper was utilized as the release device, and it was hung from the inside of the trap.

RESULTS AND DISCUSSION

Isolation and Identification of Active Compounds. *M. resinosa* pedestrian males demonstrated positive klinotaxis upon exposure to an air puff from a medicine dropper charged with solvent-or-aeration extracts of females (Park et al., 1986). While following the plume, males often raised their wings and antennae in a characteristic fashion. Bioassays of extract fractions showed that wing-raising and taxis were elicited by different compounds. Dodecanol was confirmed to be the principal component of the fraction that stimulated wing-raising, and, in bioassays, synthetic dodecanol (1 μg in 1 μl hexane) repeatedly elicited the typical wing-raising behavior.

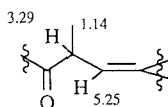
Bioassays of fractions after three successive GC fractionations placed the principal component of the sex attractant in subfraction 3-5-4 (Park et al., 1986). Spectral data suggested most aspects of the chemical structure but did not permit discrimination among possible structures 1-4. Synthesis of analogs 3 and 4 and comparison of their NMR spectra with that of the isolated attractant resulted in the assignment of structure 1 for the attractant.

Identification of Pheromone Components. Unit-resolution GC-EI MS of the isolated attractant gave the following significant peaks (%): m/z 250 (M, 0.9), 141 (4.4), 123 (24), 109 (100), 81 (20), 67 (35), 57 (21), 55 (26), 43 (57), 41 (48). A GC-CI MS (methane) gave a base peak of m/z 251, thus confirming m/z 250 as the molecular ion. The high-resolution GC-MS gave a molecular ion at 250.2268 m/z ($C_{17}H_{30}O$, index of hydrogen deficiency of three). The molecule split into two fragments: 141.1275 m/z (10%, $C_9H_{17}O$) and 109.0993 m/z (base, C_8H_{13}), the former containing a nonconjugated C=O group (GC-FTIR intense peak at 1747 cm^{-1}), the latter containing two elements of unsaturation in the form of multiple bonds or rings, and possibly branching, so disposed as to stabilize the charge.

A 500-MHz [1H]NMR spectrum, in deuterated benzene, and associated single-frequency decouplings provided the bulk of the data used in elucidating the structure(s). Although there were some impurities present in the isolated

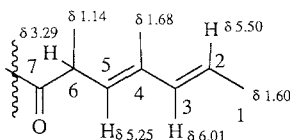
sample, the following peaks were easily discerned: δ 0.76 (d, 3H), 0.81 (d, 3H), 0.85 (d, 3H), 1.14 (d, 3H), 1.60 (d, 3H), 1.68 (s, 3H), 2.40 (t, 2H), 3.29 (m, 1H), 5.25 (d, 1H), 5.50 (m, 1H), and 6.01 (d, 1H). These absorptions account for 24 of the 30 protons and allow us to make certain tentative assumptions. The molecule contains six methyl groups, three of which are quite far upfield and are probably associated with purely aliphatic surroundings; two of them at δ 1.60 and 1.68 are probably olefinic methyls, and one of them has an intermediate chemical shift. The compound contains three olefinic protons that are probably associated with two different double bonds. Furthermore, to account for the downfield doublet at δ 6.01, the two double bonds are assumed to be conjugated.

Most of the structure can be assembled on the basis of the decoupling experiments. Irradiation of the methyl doublet at δ 1.14 collapsed the multiplet at δ 3.29 to a doublet. Irradiation of the multiplet at δ 3.29 collapsed the doublets at δ 1.14 and δ 5.25 to singlets. Since this spin system is closed (no other proton couplings), and since the downfield chemical shift of the methine multiplet at δ 3.29 cannot be explained solely on the basis of being allylic, the proton is probably next to the C=O group; thus we draw the fragment shown in Scheme 1, which contains a trisubstituted olefin.



SCHEME 1.

Irradiation of the doublet at δ 6.01 collapsed the multiplet at δ 5.50 to a quartet. Termination of this end of the chain was deduced by irradiating at δ 5.50; the doublet at δ 1.60 collapsed to a singlet. Since the two double bonds, one of which is trisubstituted while the other is disubstituted, are conjugated, the other olefinic methyl group at δ 1.68 must be on C-4. This information allowed us to piece together completely one end of the molecule, including the carbonyl group (Scheme 2). The terminal double bond can be assigned the *trans* configuration on the basis of the large (17 Hz) coupling constant between the two olefinic protons. The stereochemistry of the trisubstituted double bond cannot be determined from this information.



SCHEME 2.

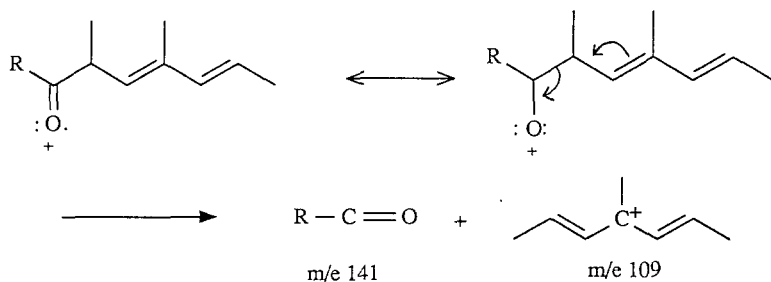


FIG. 1. Cleavage in the mass spectrum to give base peak.

The mass spectrum indicated that the main cleavage was adjacent to the carbonyl group to give, as the base peak, a hydrocarbon fragment containing a highly stabilized tertiary carbocation (m/z 109, C_8H_{13} , Figure 1). The remainder of the molecule is represented by the peak at m/z 141 ($\text{C}_9\text{H}_{17}\text{O}$, Figure 1). The β,γ -unsaturated carbonyl system accounts for all of the "functional groups" because subtraction of the formula for this fragment, $\text{C}_9\text{H}_{13}\text{O}$, from the molecular formula, $\text{C}_{17}\text{H}_{30}\text{O}$, gives C_8H_{17} —a saturated hydrocarbon chain. This chain, R in Figure 1, contains three methyl groups, each of which is adjacent to a single proton since each shows a doublet in the NMR spectrum. Individual irradiation of the methyl doublet at δ 0.81 and at δ 0.85 caused a partial collapse of the multiplet at δ 1.58, indicating that the hydrocarbon chain terminates with an isopropyl group. Only placement of the methyl group represented by the doublet at δ 0.76 remains. Unfortunately, the important methine proton adjacent to this methyl group is obscured by impurities. Therefore, the evidence can only reduce the number of possible structures to four, if one ignores the stereochemistry of the two chiral centers. The four structures are shown in Figure 2.

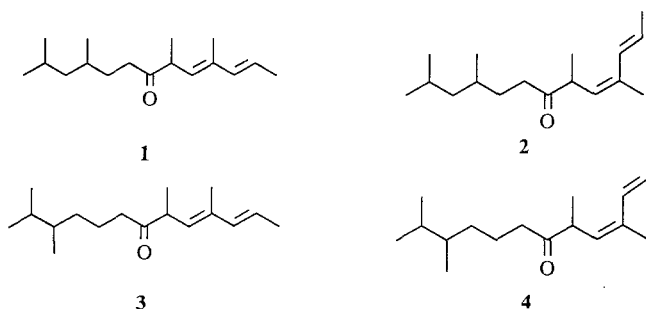
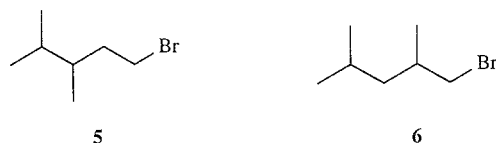


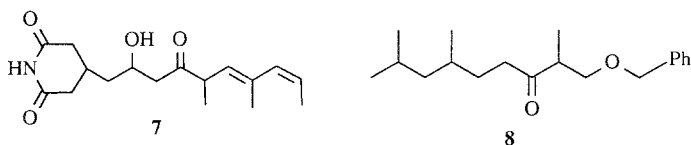
FIG. 2. The four structures permitted by the spectral data.

To complete the structure assignment, a synthetic scheme was devised, the essential features of which (1) utilize either of the two primary bromides (**5** and **6**, Scheme 3) thus enabling us to pinpoint the uncertain position of the final methyl group; and (2) provide both geometric isomers during the formation of the trisubstituted double bond.



SCHEME 3.

We now have enough information to identify the isolated attractant, having synthesized **3** and **4**. Although the methyl group is in the wrong position, the synthesis did allow us to assign the correct methyl position and the stereochemistry of the trisubstituted double bond as *trans* by NOE NMR experiments with both analogs **3** and **4**. Irradiation of the methyl group connected to the trisubstituted olefin in **3** gave no NOE enhancement while irradiation of the corresponding methyl group in **4** gave an expected NOE enhancement. The same type of enhancement was noted in a similar olefinic system by Takeda et al. (1982). A similar β,γ -unsaturated ketone system in the antibiotic 9-methylstreptimidone **7** also assisted in assigning the stereochemistry (Scheme 4) (Becker and Rickards, 1979). The greater biological activity of the *E* analog **3** over that of the *Z* analog **4** may be an additional, albeit nonrigorous, argument for the *4E* configuration of the isolated attractant (see below).



SCHEME 4.

The hydrocarbon portion of the pheromone molecule has been synthesized and analyzed. A 500-MHz ^1H NMR spectrum of the ketone/benzyl ether, **8**, closely matches the upfield portion of the ^1H NMR spectrum of the pheromone. We are now converting compound **8** into the racemic pheromone, **1**, whose systematic name is (*2E*, *4E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one and to which we assign the trivial name matsuone. Details of the synthesis of racemic compounds **1-4** will be reported elsewhere, and elucidation of the stereochemistry at C-6 and C-10 will be undertaken for compounds **1** and **2**.

Bioassays of Sex Pheromone Analogs 3 and 4. Males of *M. resinosa* were attracted by 2×10^{-4} FE of the *M. thunbergiana* female crude extract in the medicine dropper bioassay. The mean response score approximates those of *M. resinosa* and *M. thunbergiana* to 3×10^{-4} FE of *M. resinosa* female crude extract (Park et al., 1986).

At concentrations of 0.05 ng and higher, compound **3** elicited response by male *M. resinosa* equivalent to that of 2×10^{-4} FE of extract of *M. thunbergiana* females, while the index of response to **4** at a concentration 200 times higher (0.01 μ g) was, at most, 50% of that of **3** (Table 1). The threshold concentration was 5 pg for **3** and about 0.1 ng for **4**, although the mean value of response scores at this concentration was not significantly greater than that of the hexane control. A decrease in the attractiveness of **4** at concentrations higher than 0.01 μ g (Table 1) was associated with apparent irritation (movement sideways from the dropper tip or momentary cessation of walking, lowering the body and/or raising wings).

In the dummy female experiments, male behavior was classified into four categories: (1) males that showed no interest, by passing by or walking less

TABLE 1. MEAN RESPONSES OF *Matsucoccus resinosa* MALES TO DILUTIONS OF PHEROMONE ANALOGS AND CRUDE EXTRACT OF *M. thunbergiana* FEMALES^a

Material	Quantity ^b	No. tested	Mean response ^c
Hexane		23	0.04 E
Crude Extract	2×10^{-5} FE ^d	15	0.27 DE
	2×10^{-4} FE	15	2.33 A
Compound 3	0.5 pg	28	0.13 DE
	5.0 pg	28	0.70 BCD
	50.0 pg	28	2.54 A
	0.5 ng	15	2.80 A
	5.0 ng	21	2.74 A
Compound 4	10.0 pg	5	0.20 DE
	0.1 ng	21	0.57 CDE
	1.0 ng	21	0.95 BC
	10.0 ng	21	1.21 B
	0.1 μ g	21	0.93 BC
	1.0 μ g	21	0.63 BCD

^aResponse scores: 0 = no following; 0.5 = followed dropper for less than one full test triangle; 1, 2, or 3 = number of triangle sides completed.

^bEach in 1 μ l hexane.

^cMeans followed by the same letter are not significantly different ($P < 0.05$, Duncan's multiple-range test).

^dFemale equivalent.

than two female body lengths on the dummy females, (2) those that lingered on dummy females by walking on them for more than two female body lengths, (3) those that showed obvious copulation attempts, and (4) those that, upon contact with a dummy female, showed apparent irritation by retreating a short distance sideways or backwards. A male that avoided contact with dummy females often lowered its body or raised its wings. All males or those that showed avoidance reaction either walked away or returned to the dummy females and repeated the avoidance behavior.

Actively walking males, both in the presence and absence of pheromone, held their antennae parallel to the long axis of the body in lateral view and about 90° from each other in dorsal view. Wings were usually positioned flat, and the tips were dragged when walking; the costal margins of the wings made about a 90° angle with each other. The posture of the antennae or wings did not change until the male contacted the female or other substances bearing sufficient pheromone. This was in contrast with the behavior of the citrus mealybug (Gravitz and Willson, 1968) and the yellow scale (Moreno et al., 1972), where the posture of male antennae was changed before touching the females. When a male contacted and climbed on the female, the antennae closed to an angle of $0-30^\circ$ and were lifted $30-80^\circ$. The wings also were often lifted $30-60^\circ$. The male attempted a copulation thrust by bending the aedeagus downward, either immediately or after orienting his body to that of the female. This posture was maintained until copulation was completed.

Table 2 shows the results of the dummy female tests. Most males made copulatory attempts or lingered on dummy females treated with 2×10^{-3} FE of crude extract. Some males avoided dummy females bearing 2×10^{-2} FE—a concentration 100 times stronger than that which elicited strong response in the medicine dropper assay (Table 1). On the contrary, avoidance behavior was not observed when males were presented with dummy females treated with 5 ng of **3**, which is also 100 times stronger than what is required for a strong response in the medicine dropper assay. Males presented with dummy females treated with **4** did not attempt copulation and either showed no response or even avoided them. Although our female extract was made at 10 AM, some of the newly emerged females probably copulated before they were collected and immersed in hexane. Males of *M. thunbergianae* that showed avoidance behavior to the same crude extract dilutions used in this experiment, did not avoid dummy females bearing 5×10^{-2} FE of extract made from newly emerged virgin females (Park, 1988). This suggests that mated females produce a chemical that inhibits male copulatory behavior (perhaps this is the role of dodecanol). Equal parts of compounds **3** and **4** on dummy females evoked lingering and copulatory attempts by males. However, when **4** was increased relative to **3**, most males avoided the dummy females. Although the number of males tested was too small for statistical significance, and since male physiology

TABLE 2. BEHAVIOR OF *Matsucoccus resinosae* MALE PRESENTED WITH DUMMY FEMALES BEARING PHEROMONE ANALOGS OR CRUDE EXTRACT OF *M. thunbergianae* FEMALES

Material	Quantity ^a	No. tested	No. showing behavior ^b			
			N	L	C	A
Crude extract	2×10^{-3} FE ^c	8	1	3	4	0
	2×10^{-2} FE	15	2	5	6	2
Compound 3	0.5 ng	13	2	4	7	0
	5.0 ng	7	1	1	5	0
Compound 4	0.1 μ g	6	4	0	0	2
	1.0 μ g	14	5	0	0	9
Mixture 1 ^d		2	0	1	1	0
Mixture 2 ^e		15	4	2	1	8

^a Each in 1 μ l hexane.

^b N = no response; L = lingering; C = copulatory attempt; A = avoidance (see text for description of behavior).

^c Female equivalent.

^d Compound 3-compound 4-hexane = 1:1:8.

^e Compound 3-compound 4-hexane = 1:3:6.

affecting copulatory behavior should be studied further, it is apparent that pheromone analogs may elicit inhibitory as well attractive behaviors.

In laboratory bioassays with *M. matsumuræ* in China, compound 3 was significantly more attractive than compound 4 at all doses (Table 3). Qi et al.

TABLE 3. MEAN RESPONSES OF *Matsucoccus matsumuræ* TO DILUTIONS OF PHEROMONE ANALOGS 3 AND 4

Material	Dose (μ g)	$X \pm SE^a$
Compound 3	1.3	$27.7 \pm 7.03a$
	2.6	$52.7 \pm 9.30b$
	5.3	$53.7 \pm 7.66b$
	8.0	$54.7 \pm 1.76b$
	16.0	$77.6 \pm 9.13c$
	26.0	$81.0 \pm 5.27d$
Compound 4	1.3	$2.7 \pm 3.51e$
	8.0	$6.7 \pm 3.51f$
	16.0	$13.0 \pm 3.04g$

^a Means followed by the same letters are not significantly different as determined by the Wilcoxon-Mann-Whitney rank-sum test.

TABLE 4. MEAN TRAP CATCHES OF *Matsucoccus matsumuræ* MALES TO TRAPS BAITED WITH COMPOUNDS 3 AND 4

Material	Dose (μg)	$X \pm \text{SE}^a$
Compound 3	16	$2.67 \pm 1.76a$
	56	$17.33 \pm 6.33b$
Compound 4	32	$1.33 \pm 7.03a,c$
Control		$0.33 \pm 1.76c$

^aMeans followed by the same letters are not significantly different as determined by the Wilcoxon-Mann-Whitney rank-sum test.

(1983) found that 16 μg of compound 3 elicited a response comparable to 5 FE in laboratory bioassays. In field tests in China, only the 56- μg dose of compound 3 attracted significantly more males than the controls; both 32 μg of compound 4 and 16 μg of compound 3 were equivalent in attractiveness to the controls (Table 4).

Analogs have not been found previously to reduce catches of flying male scales on traps (Roelofs et al., 1977; Gieselmann et al., 1980) or close range attraction by walking males (Heath et al., 1979). It is not known whether some of these coccoid pheromone analogs previously reported have inhibitory effects on close range attraction or copulatory behavior.

Based on our results and previous reports of partial activity of coccoid pheromone analogs, we expect the synthetic pheromone (1) of *M. resinosa* to show much higher activity than its analogues; these studies are in progress.

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MECHANISM OF DAMAGE-INDUCED ALKALOID PRODUCTION IN WILD TOBACCO

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Abstract—Greenhouse-grown tobacco plants of the species *Nicotiana sylvestris* (Solanaceae) subjected to leaf damage show a fourfold increase in the alkaloid content of their undamaged leaves. This increase in nicotine and normicotine concentrations begins 19 hr after the end of the damage regime, reaches a maximum at nine days, and wanes to control levels 14 days after the start of leaf damage. The increase in leaf alkaloid content in damaged plants is largely due to a 10-fold increase in the alkaloid concentration of the xylem fluid entering leaves, which, in turn, suggests that increased synthesis of alkaloids is occurring in the roots. This research distinguishes between positive and negative cues affecting the change in xylem fluid alkaloid concentrations. A negative cue, such as auxin, when lost or diminished as a result of leaf damage could signal the alkaloidal response. Indeed, exogenous applications of auxin to damaged leaves inhibit the alkaloidal response. However, attempts to block endogenous auxin transport by steam girdling or applying an auxin transport inhibitor fail to mimic the effect of leaf damage on leaf alkaloid concentrations. The damage cue appears to be a positive cue that is related to the timing and the amount of leaf damage rather than to the amount of leaf mass lost. Moreover, when performed proximally to leaf damage, steam girdling truncates the alkaloidal response. This induced alkaloidal response appears to be triggered by a phloem-borne cue that allows the plant to distinguish between different types of leaf damage. The physiological and ecological consequences of the mechanism of this damage-induced alkaloidal response are further explored.

Key Words—Mechanism of induced defense, alkaloids, *Nicotiana sylvestris*, auxins, damage cue, nicotine, normicotine.

INTRODUCTION

Leaf damage increases the alkaloid content of undamaged leaves of *Nicotiana sylvestris* (Baldwin, 1988a–c), and this increase in leaf alkaloids reduces the growth of a tobacco herbivore (Baldwin, 1988b). Although damage-induced defenses have received much attention in the ecological literature (Rhoades, 1979; Schultz and Baldwin, 1982; Haukioja, 1982; Karban et al., 1987; Chiang et al., 1987), these responses have received little attention from plant physiologists. The mechanisms of only two herbivore-induced responses are understood in any detail: the induction of proteinase inhibitors in tomato leaves (Ryan, 1983) and the induction of quinolizidine alkaloids in lupine leaves (Wink, 1983). Ecologists have numerous hypotheses to explain why plants have inducible defenses, yet we understand little about the mechanism responsible for induction. Moreover, only by understanding the mechanism responsible for a particular trait can one assess its responsiveness to selection.

The alkaloids of tobacco—particularly nicotine—are among the best-studied secondary metabolites of plants (Tso, 1972; Leete, 1977; Waller and Nowacki, 1978). Removal of flowering heads at the onset of flowering, a standard practice in the cultivation of tobacco (*N. tabacum*), increases the size, mass, and alkaloid content of leaves (Woetz, 1955). This increase in leaf alkaloid content after removal of the flowering top is largely a result of increased nicotine synthesis in the roots (Mizusaki et al., 1973). The alkaloids are then carried to the leaves by the xylem stream (Dawson, 1942). However, decreased rates of leaf nicotine degradation in the leaves (Yoshida, 1962) and leaf nicotine synthesis (Bose et al., 1956) may also play a role in the topping-induced increase in leaf alkaloid levels. Most nicotine synthesis in cultivated tobacco is thought to occur in young dividing root tips (Dewey et al., 1955). The literature on cultivated tobacco suggests four reasonable mechanisms for the dramatic damage-induced increase in levels of leaf alkaloids in wild tobacco: (1) lower alkaloid degradation rates in leaves; (2) higher rates of alkaloid synthesis in the leaves; (3) higher transpiration rates, resulting in greater transport of alkaloids from roots to leaves; and/or (4) increased amounts of alkaloid entering the leaf in the xylem stream. The latter mechanism could be a result of higher alkaloid synthesis in those roots with xylem traces leading to undamaged leaves and/or the efficient reloading of alkaloids into these xylem traces. This, in turn, would require the extraction of alkaloid from the acidic xylem sap by transfer cells in the stem or roots and would undoubtedly be an energy-demanding process. Knowing which mechanism(s) is correct should be of great interest to ecologists, for two of these (1 and 3) could conceivably operate at little or no cost to the plant, which means the induced defense could represent merely a passive reorganization of a constitutive defense.

This research demonstrates that hypothesis 4 is the most probable expla-

nation for how the increase in alkaloids comes about. Leaf damage results in a dramatic increase in the alkaloid concentration of the xylem stream entering undamaged leaves on damaged plants. Repeated measures of alkaloid concentrations of excised leaves indicate that rates of alkaloid degradation and/or leaf alkaloid synthesis do not differ significantly between damaged and undamaged plants. Moreover, transpiration rates are not different between same-aged leaves of damaged and undamaged plants.

A distinction is drawn between a positive cue, which stimulates root alkaloid synthesis and is produced by or correlated with leaf damage, and a negative cue, which is continuously produced by leaves and when lost or diminished during leaf damage signals the induced response. A candidate for a negative cue is the plant hormone auxin, which is produced by flowering tops as by well as young leaves in *N. sylvestris* (Avery, 1935) and has demonstrated effects on nicotine synthesis (Solt, 1957; Yasumatsu, 1967; Tabata et al., 1971; Mizusaki et al., 1973; Tiburcio et al., 1985). Indeed, the alkaloidal response is completely inhibited by an application of auxins on the damaged leaf edge. However, although exogenously applied auxins clearly affect the alkaloidal response, a change in endogenous auxin transported from shoot to root is not likely to be the damage cue. Both application of an auxin transport inhibitor (TIBA) and steam-girdling, which halts the transport of endogenous auxins from leaf to root (the site of damage to the probable site of alkaloid synthesis), fail to mimic the alkaloidal response to leaf damage.

METHODS AND MATERIALS

Alkaloid Analysis and Plant Growth. The two principal alkaloids of *N. sylvestris* Spegazzini and Comes—nicotine and normicotine—were separated and quantified by high-pressure liquid chromatography with an internal standard technique (Baldwin, 1988a) for all samples except those of the time-course experiment, where an external standard technique was used. Values are expressed (mean \pm SD) as percent dry leaf weight or millimolar xylem exudate. All plants were destructively sampled. Leaf samples for alkaloid analysis were removed from the midsection of the lamina (Rosa, 1973) with a razor in a 1-cm-wide band across the leaf but excluding the midrib, and immediately extracted (Baldwin, 1988a). All leaf alkaloid analyses were performed on undamaged leaves in positions 4, 5, or 6 (from the top). Leaves in these positions on rosette-stage plants were known to exhibit large induced alkaloidal responses. A comparable band adjacent to the sample band was removed, weighed, dried at 50°C for 48 hr, and reweighed for percent dry mass calculations. In the time-course experiment, two 1-cm² circular leaf disks, one from each side of the midrib, were extracted for alkaloid analysis. The remaining

shoot mass and all leaf cuttings were placed individually in labeled bags, dried for 48 hr at 50°C, and weighed.

All plants except those used for the steam-girdling and within-plant experiments were greenhouse-grown in 8-oz. food cups with 3.5 g of Osmocote 14-14-14 fertilizer and transplanted into 18-cm fiber pots five to seven days before the start of the damage regime to avoid inhibition of the induced response as a result of hindered root growth (Baldwin, 1988a). All plants for each experiment were inbred full sibs, of the same age (by germination date), size (by length \pm 5 cm of alkaloid sample leaf, or height to developing flower bud), and clockwise phyllotaxy, and were randomly assigned to treatment groups. Moreover, plants were randomly assigned positions on the greenhouse bench. Plants used in the steam-girdling experiment were 89 days old and had developed flower buds and commenced stalk elongation. All others were in the rosette stage of growth and were 57–71 days old, depending on the experiment.

Leaf Diffusive Resistance and Water-Loss Measurements. Abaxial transpiration rates were estimated with a LiCor LI-700 transient porometer on the portions of the leaf lamina from which the alkaloid sample was later removed. The porometer was calibrated in R values ($\text{sec}/\text{cm}^{-1}$) with a porous plate technique (van Bavel et al., 1965) immediately before and after measurements. Such diffusive resistance measures are valuable for estimating leaf water-loss rates over short time intervals.

Water-loss rates from three age classes of fully expanded leaves were determined gravimetrically over four 24-hr time periods. Forty-five plants growing in 18-cm fiber pots with 505 ± 15 g of artificial soil mix were assigned to four treatment groups. All leaves in each group were removed from the plant with a razor, except those in positions 3–9 (group 1), leaves 7–9 (group 2), leaves 5 and 6 (group 3), and leaves 3 and 4 (group 4). Cut petioles were sealed with lanolin. Remaining leaves were traced at the start and end of the experiment. Leaf areas of each plant were determined by weighing the cut-out tracings, converting these masses into areas (cm^2), and calculating a mass–area regression from known areas of the remaindered tracings. A fifth treatment group consisted of nine pots with 505 ± 2 g soil, but instead of plants, it contained an 18-cm-diameter circular cardboard disk suspended 10 cm above the soil level on a dowel in each pot. This group was used to estimate the amount of water lost from the shaded soil surface. Pots were watered with 400 ml of tap water, left to drain for 30 min, weighed, and reweighed 24 hr later to 0.5 g. Values were corrected for water loss from the shaded soil surface and expressed as milligrams of water lost per square centimeter fresh leaf area per hour.

Alkaloid Sampling of Xylem Stream. Alkaloid concentration of the xylem stream of sample leaves was determined by placing the entire plant (root, soil, and shoot) into a Scholander pressure bomb (Scholander et al., 1965). Three centimeters of petiole from the sample leaf protruded from the top of the bomb.

Xylem fluid was collected at low pressures (up to 0.12 MPa) into a tared microcapillary inserted (Waldron, 1976) into the visibly distinct (Avery, 1933) xylem bundles of the petiole. Collected xylem fluid (7.6 ± 3.2 mg) was extracted into an aqueous methanol solution (0.962 ± 0.056 g) containing the internal standard and analyzed by HPLC.

Time Course of Induction Response. This experiment was designed to ascertain the time course of the plants' alkaloidal response to damage and to examine its physiological basis. Sixty plants were transplanted on February 15, and leaves in positions 5 and 6 were labeled with twist-ties around the petiole. Plants were assigned to 10 sampling groups containing six plants each. The plants in each sampling group were assigned to one of two treatment groups: control and damaged treatments. On February 21, sampling and leaf cutting commenced. Plants in the damaged treatment group had their leaves in positions 3–9 (excluding sample leaf positions 5 and 6) cut with scissors during six cuttings over a 60-hr period. Sample groups were randomly selected for analysis. Analysis of the six (three control, three treatment) plants in each sample group proceeded as follows: leaf transpiration rates were measured, and alkaloid and percent dry mass samples were removed from leaf position 5; plants from sample groups analyzed 127.5, 177.5, and 266.5 hr after the start of the experiment were placed in the pressure bomb to collect xylem fluid samples from the severed xylem bundles leading to leaf position 5; a leaf disk alkaloid sample from position 6 was removed and the leaf was cut from the stem with a razor, placed in 25 ml tap water in a controlled environmental chamber (26°C; 12:12 hr day-night cycle) for 48 hr, after which a second leaf disk alkaloid sample and percent dry mass sample were removed; the remaining shoot was placed in a labeled bag, air-dried at 50°C, and weighed; roots were irrigated over 0.2 cm² plastic mesh to remove soil, dried with paper towels, and a sample of roots was subjected to extraction of alkaloids; the remaining root mass was weighed, air-dried at 50°C, and reweighed.

Steam-Girdling Experiment. This experiment was performed to kill the phloem vascular connections between leaves and roots and to elucidate the nature of the damage cue. Steam was generated in a glass flask on a portable hot plate and directed toward the stem via foil-covered tubing that terminated in a Pasteur pipette. Tissues surrounding the girdle, which was typically 0.5 cm wide, were protected with foil. Leaf transpiration measurements on alkaloid-sample leaves located distal to the girdle were used to diagnose damage to the xylem vasculature, both before and 24 hr after girdling.

On July 15, 30 plants that had developed flower buds and commenced stalk elongation and that had their fifth leaf labeled as a sample leaf were transplanted into 30-cm pots and assigned to the five following treatment groups: untouched control plants (group 1); plants with steam-girdled stems immediately below the ninth leaf (group 2); plants girdled as in group 2 and with leaves in positions

4–9 (excluding sample position 5), i.e., those above the girdle, cut with scissors over a 60-hr period (group 3); plants damaged as in group 3 but not steam girdled (group 4); and plants girdled as in group 2 and with leaves in positions 10–14, i.e., those below the girdle, cut with scissors over a 60-hr period. The position 5 leaf was sampled for alkaloids 100 hr after the start of the leaf damage.

Timing of Leaf Damage. This experiment was designed to determine the effect of the timing of leaf damage on the induction response when the number and age of leaves removed were held constant. Forty-five plants were transplanted on January 7. Plants were assigned to three treatment groups: a control group, to be sampled only for leaf alkaloids; a sudden-damage group, in which leaves in positions 3–9 (excluding sample leaves in position 4) were cut at each leaf's attachment to the stem with scissors on January 10 (defining the start of the experiment); and a gradual-damage group, in which leaves in the same positions were cut in quarters (by leaf length) at times 0, 31, 48, and 52 hr after the start of the experiment, so that by 52 hr the same amount of leaf area from the same leaf positions had been removed from both damage groups. Three plants from each treatment group were randomly chosen for alkaloid analysis at 69, 76, 93, 117, and 142 hr after the start of the experiment.

Auxin Inhibition of Induction Response. This experiment was designed to determine: (1) the effects of auxins—indole-3-acetic acid (IAA) and naphthalene-1-acetic acid (NAA)—applied to the damaged leaf edge on the induction response, and (2) the ability of an inhibitor of polar auxin transport (Nieder-gang-Kamlen and Skoug, 1956)—2, 3, 5-triiodobenzoic acid (TIBA)—to mimic the effect of leaf damage. NAA, IAA, and TIBA were made up fresh daily in lanolin (0.1% by mass). Leaf cutting followed the format of the time-course experiment. Thirty-six plants were transplanted on June 7. After having their alkaloid sample leaves labeled, they were assigned to six treatment groups with six plants per group as follows: (1) control plants sampled for alkaloids only; (2) auxin-control plants with leaves at positions 5–9 (excluding sample leaf 6), which were cut with scissors and the cut edges then thinly coated with a layer of lanolin; (3) undamaged TIBA-control plants that had a thin band of lanolin spread on the base of their leaves at positions 5–9 (except position 6, the alkaloid sample position) after each leaf cutting; (4) NAA-treated plants that were damaged as in group 2, and the cut edges were thinly coated with a layer of NAA-containing lanolin; (5) IAA-treated plants that were treated like those in group 4 but had IAA-containing lanolin spread on cut leaf edges; and (6) TIBA-treated plants that were treated like those in group 3 but had TIBA-containing lanolin spread on the base of the leaves. All plants were harvested for alkaloid analysis and dry mass determinations 120 hr after the start of leaf cuttings.

Transpiration Inhibition of Alkaloid Transport. Previous experiments with plants in gas-tight Plexiglas chambers growing in CO₂-free (0–10 ppm) air and

330 ppm CO₂ air for 10 days demonstrated that plant growth conditions below the CO₂ compensation point had little effect on leaf alkaloid content, measured as percent of leaf dry mass (Baldwin, unpublished). This result allowed the development of an experiment that reduced the transpiration rates of leaves and hence the transport rates of alkaloids into sample leaves by enclosing sample leaves in clear polyethylene bags. Twenty-four plants were transplanted on August 1. On August 6, sample leaves at positions 5 and 6 were labeled; all plants were cut with scissors at positions 3–9, excluding sample leaf positions 5 and 6, over a 50-hr period and were subsequently assigned to four treatment groups: (1) plants without bags; (2) plants with bags on leaf position 5; (3) plants with bags on leaf position 6; (4) plants with bags on leaf positions 5 and 6. Bags were removed at dusk and replaced at dawn. All sample leaf positions were removed for alkaloid analysis 96 hr after the start of the damage regime.

Within-Plant Distribution of Alkaloids. Twelve plants were grown in 2.5-cm × 8-cm sections of plastic pipe and transplanted June 30 to 6.4-cm wide × 14-cm sections of pipe to facilitate placement of plants into the Scholander bomb without removing plants from the pots. Leaves at positions 3, 4, 8, and 9 were labeled as sample leaves, and plants were assigned to two treatments: (1) control plants that were undamaged, and (2) damaged plants that had leaves at positions 2–12 (excluding the alkaloid sample positions) cut over a 48-hr period. Leaf transpiration measures and leaf and xylem fluid alkaloid samples were taken from the four sample leaf positions on all plants 105 hr after the start of leaf damage.

Statistical Analysis. Alkaloid values and plant masses from each experiment were *N* scored (Ryan et al., 1982) and tested for statistical normality. If the data were normally distributed, treatments were tested with a one-way ANOVA; if not, they were compared with the Mann-Whitney U test (Sokal and Rohlf, 1981). Percentages were arcsine transformed for parametric statistical analysis.

RESULTS

Time Course of Induction Response. Plants subjected to the damage regime had high total leaf alkaloids 19 hr after the end of leaf damage regime and lost $38.6 \pm 9.9\%$ of their total shoot dry mass. This leaf alkaloid increase continued nine days (218 hr) into the experiment, reaching a maximum at 4.9 times the alkaloid content of undamaged plants; thereafter the response waned (Figure 1). Leaf alkaloid contents of undamaged plants did not change significantly ($F_{9,20} = 0.3$, $P > 0.75$), but those of damaged plants clearly did change ($F_{9,20} = 9.3$, $P < 0.0001$) over the experimental period. Nicotine represented about 95% of the total alkaloid content, and nornicotine made up the majority of the

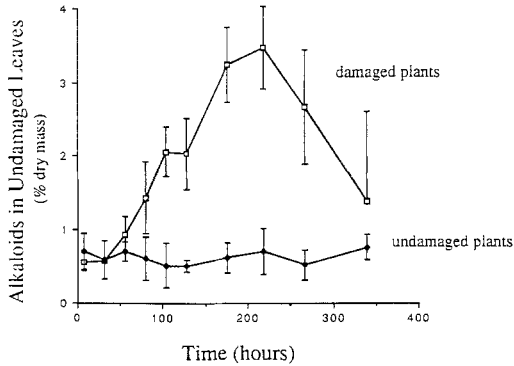


FIG. 1. Total alkaloid contents (mean % dry mass \pm SD) from leaves at stalk position 5 of 30 damaged (\square) and 30 undamaged (\blacklozenge) *N. sylvestris* plants randomly sampled, six plants per sampling time, over 338 hr from the start of leaf damage. Leaves were cut from 0 to 60 hr and their removal accounted for $38.6 \pm 9.9\%$ of the shoot mass.

remainder. Nicotine contents generally mirrored the nicotine content. The ratio of leaf nicotine to nicotine increased slightly, from 0.01 to 0.09 over the 14 days of the experiment, but this ratio did not differ significantly in damaged and undamaged plants over the duration of the experiment ($F_{9,20} = 0.6$, $P > 0.70$).

Alkaloid contents of roots, which consisted entirely of nicotine, were substantially lower (mean of all samples = $0.27 \pm 0.19\%$ dry mass) than leaf alkaloid contents and did not differ significantly between damaged and undamaged plants ($F_{6,14} = 1.2$, $P > 0.25$).

The change in leaf alkaloid contents over a 48-hr period in excised and water stem-fed leaves did not differ between leaves from damaged and undamaged plants throughout the course of the experiment ($F_{6,14} = 1.1$, $P > 0.4$). Hence, there was no evidence for differences in rates of either alkaloid synthesis or degradation in the excised leaves of damaged compared to undamaged plants. The average change in leaf alkaloids of excised leaves from both damaged and undamaged plants during the period of alkaloid accumulation (from 0 to 218 hr; Figure 1) was a decrease of $1.57 \times 10^{-3}\%$ dry mass alkaloids/hr, indicating that alkaloid degradation predominates in these leaves. If degradation were the only process responsible for changes in leaf alkaloids, damaged and undamaged plants would have the same alkaloid concentrations. Moreover, the maximum change in alkaloid levels over the experimental period would have been a decrease of about $\frac{1}{2}$ rather than the observed $4\times$ increase.

No evidence was found for compensatory growth in damaged plants; the total shoot biomass (removed plus remaining) of damaged and undamaged plants was not significantly different ($F_{7,16} = 1.4$, $P > 0.25$).

The concentration of nicotine, the only alkaloid found, in the xylem fluid of damaged plants was about 10 times higher ($F_{1,15} = 24.4$, $P < 0.0001$) than that in undamaged plants (Figure 2) and tended to wane as leaf alkaloid levels waned (Figure 1). These concentrations are slightly lower than measures of xylem fluid exuding from cultivated tobacco stumps (Dawson, 1941).

Leaf Diffusive Resistance and Water-Loss Rates. Leaf diffusive resistance was not different among treatment groups in all experiments as long as leaves occupying similar stalk positions were compared. Transpiration rates between leaves on a plant, particularly on sunny days, were different and followed the pattern established (Begg and Turner, 1970; Sasaki and Hirala, 1971) for *N. tabacum*, where diffusive resistance was lower in younger leaves.

Leaf water-loss rates measured over 24-hr periods corroborated the leaf resistance measures. Loss rates were significantly different ($F_{3,129} = 23.7$, $P < 0.0001$) between days, ranging from 3.8 ± 1.9 to 6.7 ± 1.0 mg/cm²/hr for all treatment groups. On days of low water use (3.8 ± 1.9 mg/cm²/hr) no significant difference was found between treatment groups ($F_{3,32} = 1.7$, $P = 0.20$), but on high water-use days (6.7 ± 1.0 mg/cm²/hr), treatment groups (Table 1) differed significantly ($F_{3,32} = 10.9$, $P < 0.001$); younger leaves had greater loss rates than older leaves. Not unexpectedly, daytime loss rates were higher (reaching as high as 30 mg/cm²/hr) than nighttime water loss rates.

Steam-Girdling Experiment. Plants in the different treatment groups of the steam-girdling experiment had significantly different ($F_{4,25} = 40.5$, $P < 0.0001$) alkaloid contents (Figure 3). Steam-girdled plants had leaf alkaloid contents higher than those of control ungirdled plants, indicating that girdling itself elic-

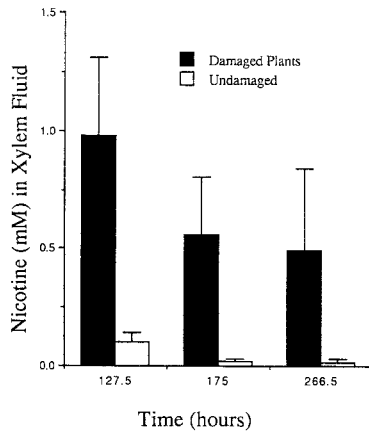


FIG. 2. Concentration (mean mM \pm SD) of nicotine in the expressed xylem fluid from xylem bundles entering sample leaves at stalk position 5 of the plants sampled in Figure 1 at 127.5, 177, and 266.5 hr after start of leaf damage.

TABLE 1. WATER-LOSS RATES FROM PLANTS WITH LEAVES OCCUPYING SPECIFIED STALK POSITIONS (LABELED TOP TO BOTTOM) ON HIGH WATER-LOSS 24-hr DAY ($6.7 \pm 1.0 \text{ mg/cm}^2/\text{hr}$)

Plants with leaves at stalk positions	Water ($\text{mg/cm}^2/\text{hr}$)	
	Mean	SD
3-9	5.8	1.0
7-9	6.0	0.8
5-6	7.0	0.6
3-4	7.7	0.6

its a small alkaloidal response; however, plants girdled and suffering leaf damage above the girdle had alkaloid contents ($F_{1,10} = 0.6$) similar to those plants only girdled. Moreover, nongirdled plants suffering similar amounts of leaf damage had significantly ($F_{1,10} = 25.2$) elevated alkaloid levels, and girdled plants that were damaged below the girdle also had elevated alkaloid levels. Steam-girdling apparently blocks the alkaloidal response to damage.

Timing of Leaf Damage. Plants in the sudden-damage group, which had their leaf material removed in a single cutting ($0.315 \pm 0.076 \text{ g}$), had the same absolute amount ($F_{1,18} = 0.8$, $P > 0.75$) and percentage of shoot mass ($F_{1,18} = 1.9$, $P > 0.5$) removed as did those plants suffering gradual damage to the same leaf positions over 52 hr ($0.349 \pm 0.088 \text{ g}$).

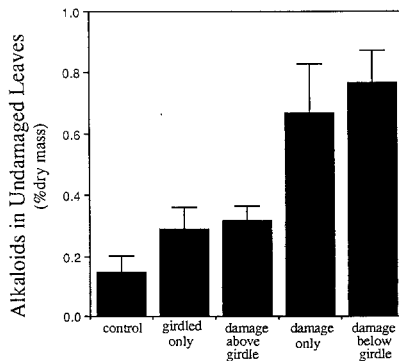


FIG. 3. Total alkaloid content (mean % dry mass \pm SD) of sample leaves occupying stalk position 5 of 30 plants (six per treatment) in the following leaf damage and steam-girdling treatments: control, undamaged, and ungirdled plants; plants girdled below the ninth leaf; girdled plants with leaves at positions 4 and 6-9 cut; ungirdled plants with leaves at positions 4 and 6-9 cut; girdled plants with leaves at positions 10-14 cut.

Although the plants in the two damage groups had the same amount of leaf mass removed from the same leaf positions, the alkaloid contents of their respective undamaged sample leaves were remarkably different (Figure 4) in all but the first sampling. Plants subjected to gradual leaf damage had an alkaloid profile that continued to rise throughout the 142-hr experiment, reaching 2.6 times that of control undamaged plants. In contrast, plants subjected to a single damage event had a significantly more attenuated response: 76 hr after damage the leaf alkaloid contents reached a maximum (at 1.4 times that of control plants) that then waned, reaching control levels by the 117-hr sampling. Plants damaged in four cuttings incurred at least four times the amount of cellular damage as those plants cut once; hence this experiment demonstrates that the alkaloidal response is influenced by the timing and the amount of cellular damage, rather than the amount of leaf area lost.

Auxin Inhibition of Induction Response. The amount of leaf mass (3.15 ± 0.58 g) or the percentage of shoot mass ($55.7 \pm 4.9\%$) removed from the plants in the three damaged-plant groups was not significantly different ($F_{2,15} = 0.4$ and $F_{2,15} = 0.1$, respectively). However, the sample leaf alkaloid contents among the six treatment groups did differ significantly ($F_{5,30} = 16.7$, $P < 0.0001$). Plants subjected to leaf damage and pure lanolin treatments had approximately four times the leaf alkaloid content of undamaged control plants and two to three times the alkaloid contents of both groups of damaged plants that had their damaged leaf edges treated with either auxin (Figure 5). Clearly,

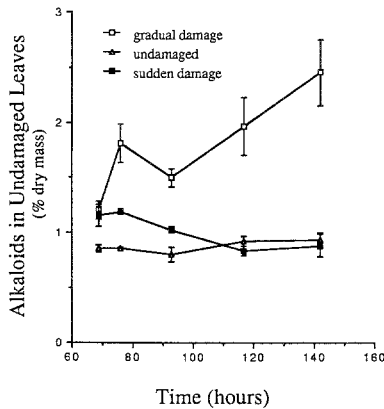


FIG. 4. Total alkaloid contents (mean % dry mass \pm SD) from stalk position 4 leaves of 45 plants in three treatment groups (three plants per treatment per sampling): control undamaged plants (Δ), suddenly damaged plants (\blacksquare) suffering leaf damage in a single cutting at time 0, and gradually damaged plants (\square) that were damaged in four cuttings from 0 to 52 hr. Plants from the two damage treatments had the same amount of leaf area removed from the same leaf positions.

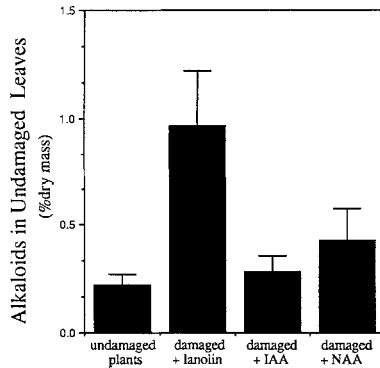


FIG. 5. Total alkaloid contents (mean % dry mass \pm SD) from leaves in stalk position 6 of 24 plants (six per treatment) in the following four treatments 120 hr after the start of leaf damage: control undamaged plants, and three damage treatments where four leaf positions were cut six times over a 60-hr period and after each cutting either pure lanolin, or lanolin with 0.1% (by mass) NAA or IAA was coated on the cut leaf edges.

the application of both synthetic (NAA) and natural (IAA) auxins to damaged leaves effectively terminates the damage-induced alkaloidal response. The application of an auxin transport inhibitor had no significant effect ($F_{1,10} = 1.7$) on sample leaf alkaloid contents (0.17 ± 0.05) compared with the alkaloid contents of plants girdled with pure lanolin (0.26 ± 0.16).

Transpiration Inhibition of Alkaloid Transport. Plastic bags on the undamaged leaves of damaged plants quickly saturated the air surrounding these leaves with water vapor and likely slowed the xylem transport rates of the bagged leaves. Leaves were not visibly affected by bagging but likely experienced higher daytime temperatures than unbagged leaves. The average alkaloid concentrations of leaves in positions 5 and 6 (1.25 ± 0.38) from damaged plants without bags were significantly ($P < 0.005$) higher ($F_{1,10} = 16.6$) than those of leaves in positions 5 and 6 (0.58 ± 0.16) of damaged plants where both sample leaves were bagged. Moreover, plants with either, but not both, leaves in positions 5 or 6 in bags, had significantly ($P < 0.0001$) higher ($F_{1,21} = 32.9$) alkaloid contents in the leaves without bags (1.13 ± 0.27) than in those with bags (0.61 ± 0.13). These results are consistent with the hypothesis that leaf transpiration rates will affect the induction response.

Within-Plant Distribution of Alkaloids. Leaf alkaloid concentrations from damaged plants were significantly higher (all $F_{1,10}$ s > 13.6 , P s < 0.005) than those from undamaged plants for all leaf positions (Figure 6). Similarly, the concentrations of nicotine—again the only alkaloid found—were approximately 10 times as high (all $F_{1,10}$ s > 11.1 , P s < 0.01) in the expressed xylem fluid

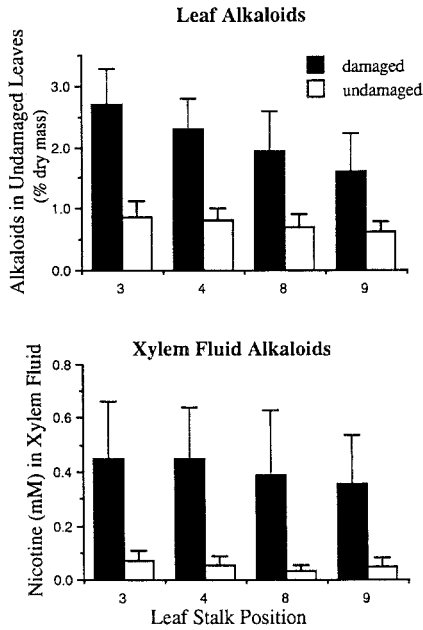


FIG. 6. Total leaf alkaloid contents (mean % dry mass \pm SD) (top) and concentration (mean mM \pm SD) of nicotine in expressed xylem fluid (bottom) from leaves in stalk position 3, 4, 8, and 9 (numbered top to bottom) from six damaged and six undamaged plants.

of damaged plants than in that of undamaged plants for all leaf positions. Interestingly, the concentrations of nicotine in the fluid expressed from the xylem bundles of the different leaf positions did not differ significantly in either damaged ($F_{3,17} = 1.9, P > 0.10$) or undamaged plants ($F_{3,19} = 1.7, P > 0.10$). The process responsible for the varying alkaloid concentrations between leaves in tobacco plants (Srivastava, 1983) (Figure 6) appears not to be the process that increases alkaloid levels after damage.

DISCUSSION

Leaf damage dramatically increases the nicotine concentration of the xylem fluid entering undamaged leaves (Figures 2 and 6). Because I have measured the rate of water loss by plants, the concentrations of alkaloids in the xylem stream entering leaves on damaged and control plants, and the rate of change in leaf alkaloids after damage, I can calculate the amount of alkaloid entering the leaf and compare this with the observed change in leaf alkaloid concentra-

tion. A damaged plant can increase its leaf alkaloid concentration from approximately 0.7 to 3.4% in 200 hr, while undamaged plants remain approximately unchanged (Figure 1). For a 1-cm² leaf disk, this represents an addition of approximately 95 μ g of nicotine in the damaged plant. A leaf in stalk position 5 uses approximately 5.5 mg H₂O/cm²/hr and thus in 200 hr will have transpired 1.1 ml of xylem fluid per square centimeter. The concentration of nicotine in the xylem fluid is likely to be 0.53 mM; thus over the 200-hr time period after the start of leaf damage, approximately 94 μ g of nicotine will have been transported to a 1-cm² area of leaf. These calculations should be viewed as rough approximations for two reasons: (1) average values were used and substantial variability in the parameters exists both through time and between plants, and (2) all the parameters were not all measured in the same experiment. Given these caveats, the calculations suggest that the amount of alkaloid entering leaves is sufficient, assuming no alkaloid turnover, to account for the dramatic change in alkaloid concentration in damaged plants.

This research demonstrates that neither alkaloid synthesis nor degradation rates in excised leaves differ between damaged and undamaged plants. However, observations from numerous tracer studies have demonstrated that nicotine is metabolically very active, with a half-life of approximately 20 hr (Leete and Bell, 1959; Yoshida, 1962) in cultivated tobacco. If nicotine is as metabolically labile in *N. sylvestris* as it is in cultivated tobacco, then either the above calculations underestimate the amount of nicotine imported into a leaf and/or the changes in alkaloid concentrations in excised leaves measured in this study do not accurately reflect the changes in intact plants—as is the case in other alkaloid-containing plants (Daddona et al., 1976).

At least two mechanisms are potentially responsible for the dramatic increase in alkaloid concentrations of the xylem fluid in damaged plants: (1) increased root alkaloid synthesis, and/or (2) altered loading of alkaloids into those xylem conduits that lead to undamaged leaves without a corresponding increase in root alkaloid synthesis. Four types of evidence indicate that increased root alkaloid synthesis contributes to the increase in xylem alkaloids. First, the administration of dye to selected root bundles of hydroponically grown plants results in dye transport to specific portions of the shoot, indicating that specific leaves have low-resistance xylem connections to specific roots. Leaf damage does not alter the pattern of dye transport (Baldwin, unpublished results). Second, no significant correlation was found in any of the experiments in this study between the amount or percent of leaf mass removed or remaining and leaf alkaloid concentration, as would be expected if roots were producing unaltered amounts of alkaloid and simply distributing this alkaloid to fewer leaves of damaged plants. Third, decapitation of cultivated tobacco dramatically increases root alkaloid synthesis (Mizusaki et al., 1973; Saunders and Bush, 1979), and it is plausible that the alkaloidal responses that result from topping and leaf

damage share a similar physiological basis. Last, the results of experiments elucidating the nature of the damage cue (Figures 3–5) are difficult to reconcile with mechanisms that do not involve increases in root alkaloid synthesis with damage.

Two lines of evidence suggest that a positive cue is the best candidate for the damage cue(s) effecting the observed change in alkaloid concentrations: the observations that (1) the alkaloidal response is clearly proportional to the timing and amount of leaf damage rather than to the amount of leaf mass removed (Figure 4), and (2) killing the phloem by steam-girdling proximally to leaf damage attenuates the alkaloidal response to damage (Figure 3).

Auxin, a likely candidate for a negative cue, has a pronounced effect on the induced response when applied exogenously (Figure 5), but attempts to block endogenous auxin movement from root to shoot failed to mimic the alkaloidal response to damage. Ethylene is known to be produced after leaf damage (Abeles, 1973) and is a potent inhibitor of auxin transport (Audus, 1972); however, foliar application of 2-chloroethylphosphonic acid—which releases ethylene into plant cells—to cultivated tobacco (Cutler and Gaines, 1971; Kasperbauer and Hamilton, 1978) or *N. sylvestris* (Baldwin, unpublished results) has little or no effect on leaf alkaloid contents. Moreover, the steam-girdling experiment (Figure 3) strongly suggests that the damage cue is phloem-borne rather than gaseous. These results do not deny that auxin may induce the enzymes involved in nicotine biosynthesis: they merely demonstrate that auxin is not functioning as a negative cue for the activation of these enzymes.

In the time-course experiments, the rise in alkaloid content of undamaged leaves after damage is rather slow (Figure 1), compared to that of other damage-induced phytochemical responses (Carroll and Hoffman, 1980). This time delay, possibly a result of the large spatial separation of the sites of damage and alkaloid synthesis, can be divided into three components: (1) delays due to transport of cue from damaged leaves to roots; (2) delays due to the activation of enzymes involved in nicotine synthesis; and (3) delays due to the loading of alkaloids into the xylem stream and their transport to the leaves.

Nicotine biosynthesis in the roots of cultivated tobacco involves the condensation of a pyridine and a *N*-methylpyrrolidine ring. The principal regulatory enzymes in the biosynthesis of these two rings are quinolinic acid phosphoribosyltransferase (EC 2.4.2.19) and putrescine methyltransferase (EC 2.1.1.53), respectively (Wagner et al., 1986a, b; Feth et al., 1986). Both of these key enzymes are substantially induced in the roots of cultivated tobacco within 24–40 hr of decapitation (Mizusaki et al., 1973; Saunders and Bush, 1979; Wagner et al., 1986a). In so far as alkaloid induction by leaf damage and decapitation comes about by a similar mechanism, these results suggest that delays that are the result of components 1 and 2 are less than 24 hr for small plants.

This research demonstrates that altered transport rates of alkaloid due to

increases in leaf transpiration are not responsible for the increase in leaf alkaloid concentrations in damaged plants. However, transpiration rates nevertheless may play an important role in how the physical environment influences this induced response. Conditions of low evapotranspiration (high humidity and low temperatures) will greatly slow both the water loss from plants and the transport of alkaloids to the leaves. The results from the transpiration-inhibition experiment are consistent with this hypothesis. Since any inhibition of transpiration will also increase leaf temperature, the results of this experiment may be confounded by temperature effects. Moreover, because nicotine concentrations of 0.5 mM—the concentration found in xylem fluid of damaged plants—in the roots will repress putrescine methyltransferase activity by at least 50% (Mizusaki et al., 1973), conditions of low evapotranspiration may actually truncate the induced defense by slowing the export of alkaloids from the sites of synthesis. An ecological prediction from this hypothesis is that this response will be most pronounced and hence more effective defensively for plants growing under conditions of high evapotranspiration. Indeed, most members of the genus *Nicotiana* are native to xeric habitats (Kostoff, 1941).

Alkaloid contents in the younger leaves occupying higher stalk positions are higher than those in older, lower leaves (Figure 6) (Srivastava, 1983), although the opposite pattern was also reported (Vickery, 1961). This within-plant distribution of alkaloids is consistent with the predictions of optimal defense theory (McKey, 1974, 1979; Rhoades, 1979; Krischik and Denno, 1983), according to which younger, photosynthetically more valuable tissues receive a greater defense allocation. The process by which this apparently optimal distribution of alkaloids comes about differs from the one responsible for the damage-induced pattern, in that leaf transpiration rate influences the within-plant distribution of alkaloid. The concentration of nicotine in xylem fluid expressed from lower-leaf xylem traces resembles that from upper leaf traces (Figure 6), but the amount of water lost per leaf area from upper leaves is significantly greater than that from lower leaf positions (Table 1). Thus the pattern of higher alkaloid contents in younger leaves is preserved in damaged plants even though the concentration of alkaloids entering the leaf is increased 10-fold. Tobacco plants appear to have two systems for affecting the optimal distribution of defense: a damage-inducible system that triggers an increase in root alkaloid synthesis and a “plumbing system” that ensures greater transport of alkaloid-containing xylem fluid to younger leaves.

Clearly, the induced defense is an active response rather than a passive deterioration of foliage quality after herbivore attack (Myers and Williams, 1984). Its mechanistic basis enables a plant to differentiate between leaf damage resulting from biotic agents, which inflict damage over a sustained period of time, and abiotic agents (leaf and branch fall or hailstorms), which inflict dam-

age that, although it may be significant, is momentary, and to which a defensive response would be inappropriate. Moreover, since the chemical changes were observed also in undamaged leaves, they are not likely to be solely a product of wound metabolism or directed only at potential microbial or viral invasion at wound sites. However, this alkaloidal response should not be viewed as purely a defensive response.

As the metabolic tracer experiments underscore, alkaloidal defense in plants must be considered an integral part of a plant's primary metabolism. Nicotine, when degraded in a cultivated tobacco plant, is metabolized to amino acids, pigments, sugars, and organic acids (Tso and Jeffery, 1959, 1961), and serves as a methyl donor to choline (Leete and Bell, 1959). The amount of nicotine (which is 17% nitrogen) transported to a leaf after damage represents a substantial fraction of the total leaf nitrogen and can be made available for other metabolic demands. Damaged plants undoubtedly have higher leaf nitrogen demands in order to support the rise in photosynthetic rates and other compensatory metabolic changes typically observed after topping in tobacco (Darkanbaev et al., 1962; Yoshinori et al., 1967) and after leaf damage in other species (Crawley, 1983). In addition, damaged plants may have other defenses, such as proteinase inhibitors (Wong et al., 1976) induced by leaf damage. All biochemical and photobiological processes of photosynthesis require nitrogenous compounds, and leaf photosynthetic rates are generally positively correlated with leaf nitrogen contents (Field and Mooney, 1986). Thus nicotine, in addition to playing an important role in leaf defense, may serve as a well-defended vehicle for nitrogen transport from roots to leaves, a transport route vulnerable to attack by the guild of sap-sucking herbivores.

In summary, the mechanistic details of the increase in leaf alkaloid concentrations after damage support the conclusion that the response is an integral part of a plant's primary metabolism and is under both strong environmental constraints (including soil nitrogen availability, water, and depth and climatic factors affecting evapotranspiration) and plant architectural constraints (including the distance between sites of damage and alkaloid synthesis, as well as the number and distribution of reproductive buds or other rich sources of auxin). These considerations will profoundly influence the evolution of the defensive role of this damage-induced change in alkaloid concentration.

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VOLATILE COMPONENTS OF ANAL GLAND
SECRETION OF AARDWOLF
(*Proteles cristatus*)

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Abstract—Volatile constituents of the anal gland secretion and of scent marks of the aardwolf were identified using dynamic solvent effect sampling, capillary gas-liquid chromatography, and mass spectrometry. Both scent marks and gland contents consisted mainly of short- to medium-chain fatty acids, a complex series of medium- and long-chain esters, indole, and hexanol. There were marked individual differences in the relative concentrations of the various components.

Key Words—Aardwolf, *Proteles cristatus*, anal gland, fatty acids, esters, territory, dynamic solvent effect.

INTRODUCTION

The aardwolf (*Proteles cristatus*) is the only member of the family Proteleidae (Meester et al., 1986). Although it shares many features with the Hyaenidae and has often been classified in that family, it is much smaller (9–11 kg) than the hyaenas and is entirely insectivorous, specializing on termites, especially *Trinervitermes* spp. (Kruuk and Sands, 1972; Cooper and Skinner, 1979; Smithers, 1983; Richardson, 1987a). Its range is divided into two main areas, one in eastern and northeastern Africa and the other in the southern African subregion. Aardwolves live in monogamous pairs, which defend territories of

1–3 km², whose size appears to be determined by the density of termite nests (Richardson, 1987b). Territories are demarcated by scent marks produced by wiping the secretion of the well-developed anal gland onto grass stems and other vegetation (Smithers, 1983; Richardson, 1985). It has been suggested by Richardson (1987b) that pastings may also be used by both sexes to attract mates and by males to intimidate other males in competition for mating opportunities.

We report here on the identities of the major volatile constituents of the anal gland secretion of male and female aardwolves.

METHODS AND MATERIALS

Adult aardwolves were captured at Benfontein game farm in the northern Cape Province, South Africa. The animals were anesthetized with ketamine hydrochloride and, by careful pressure, their anal glands were everted and the contents expressed directly into clean glass vials with polytetrafluoroethylene caps. Fresh scent marks were collected into similar vials by clipping the grass stems on which they had been deposited. All material was stored at -10°C until analysis.

Volatiles were sampled from approximately 5-mg blobs of anal gland contents of about the same volume as the real scent marks, which were sampled whole. Palladium-cell-purified hydrogen was passed over the specimens at a flow rate of 15 cm³/min and a temperature of 22–23°C. Volatiles were accumulated by dynamic solvent effect (Apps et al., 1987) using alumina-purified *n*-hexane as solvent. Sampling periods of 5–20 min were used for gas chromatography–flame ionization detector (GC-FID) analyses and 30 min for GC–mass spectrometer (GC-MS) analyses.

GC-FID analyses were carried out on a Varian 3700 gas chromatograph fitted with a dynamic solvent effect inlet (Apps et al., 1987) with a 25-m \times 0.3-mm \times 0.4- μm methyl silicone capillary column. The carrier gas was hydrogen with a linear velocity of 55 cm/sec. The starting temperature of both inlet and column was 40°C. The inlet was heated ballistically to 220°C after 2.2 min, and the column temperature was programmed at 4°C/min after 7 min. The detector sensitivity was 4×10^{-11} A/mV full scale deflection. GC-MS analyses were carried out under equivalent conditions on a modified Varian 1400, using helium as carrier gas, with an open split interface to a VG Micromass 16F spectrometer operating in the electron impact mode. The source temperature was 220°C, and the electron energy was 70 eV.

Compounds were identified by comparison of their mass spectra with those in libraries or the literature. Where necessary, identities were confirmed by retention indices and syntheses of authentic compounds.

RESULTS

The anal gland of the aardwolf contains a bright or pale orange paste, which at room temperature has the consistency of soft butter. The scent marks are blobs or smears of waxy material about 1.0 cm long varying in color from yellow to dark brown. Pale scent marks, but not anal gland contents, darken after one to three days of exposure to air. When fresh, the odor of the anal gland contents is dominated by a penetrating metallic scent. Cheesy and faint, sweet, musky odors are just distinguishable. After 10–20 min of exposure to air, the smell changes to one in which the cheesy and musky scents are dominant; after an hour the original smell returns and persists for at least six months. The odor of scent marks resembled that of fresh and/or aged gland contents. To the human nose, there were only minor differences in odor between material from males and females. The volatiles emitted in the largest quantities by both gland contents and scent marks were fatty acids (Figure 1, Table 1). In addition, there was a fairly complex mixture of minor components dominated by series of esters of the fatty acids. The only other identified compounds were hexanol and indole.

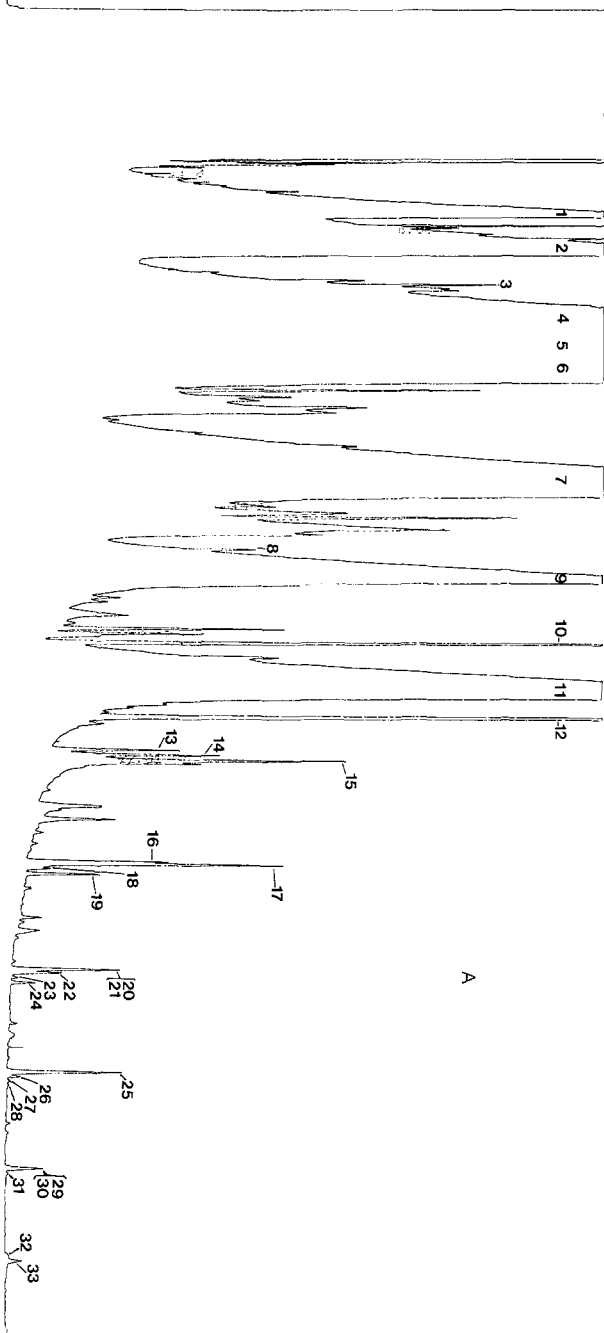
Even within the small number of specimens analyzed, there were marked differences in the occurrence and relative areas of various peaks (e.g., Figure 1). Whether these differences reflect individual identity or are related to, e.g., sex or reproductive condition, is uncertain at this stage. The role of scent marking in reproductive competition among males (Richardson, 1987b) suggests that male scent marks are systematically different to those of females.

DISCUSSION

The color of aardwolf anal gland secretion has been variously reported as dark brown, yellowish ochre, and orange (Smithers, 1983, p. 349), a variability that may be accounted for by the marked differences in color, noted here, between anal gland contents and scent marks. Although there was no systematic difference in volatile components between scent marks and gland contents, the spontaneous darkening of scent marks suggests that some chemical changes are undergone by the gland secretion shortly before its deposition as a scent mark.

The rather rapid changes in the odor of the anal gland contents, and presumably in that of very fresh scent marks, have the potential of acting as short-term indicators of an animal's movements, while the extreme persistence of the odor might be expected to enhance its effectiveness in demarcating territories.

Volatile fatty acids have a widespread, if somewhat sporadic, occurrence in mammalian secretions (Albone, 1984). Whether or not they occur generally



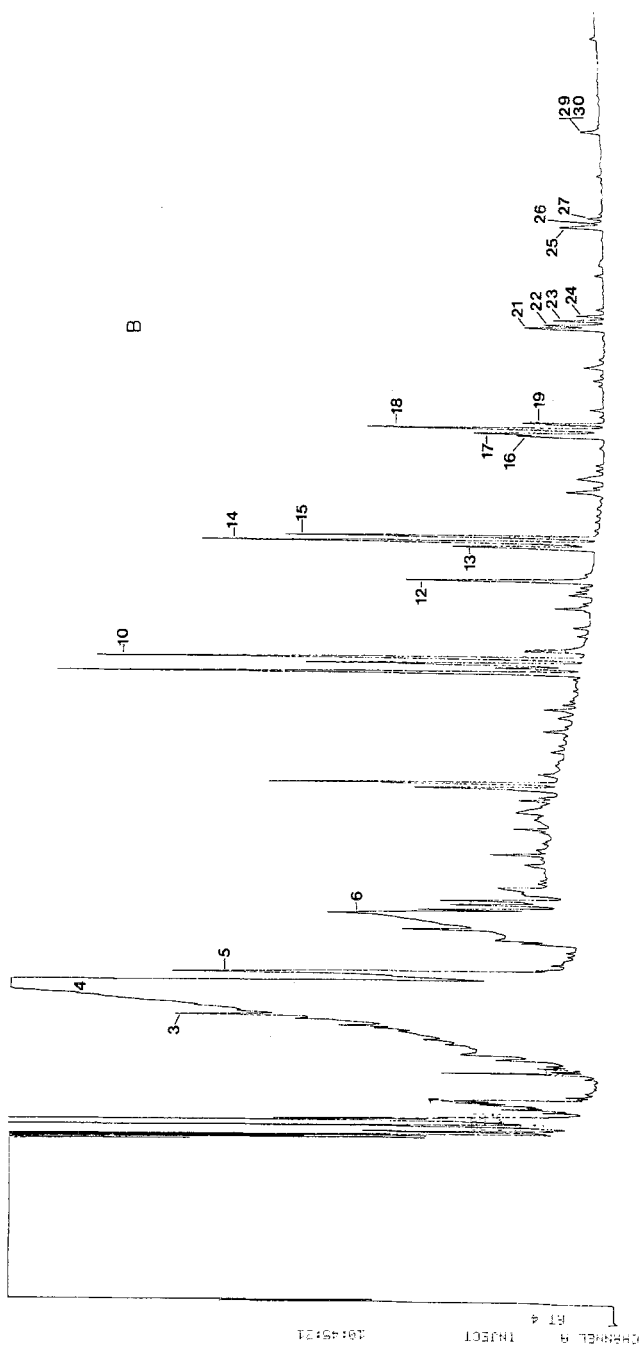


FIG. 1. Chromatograms of volatiles from anal gland contents of two aardwolves (*Proteles cristatus*). The differences between the two chromatograms give an impression of the type of variability encountered within a small set of specimens. Column: 25 m \times 0.3 mm \times 0.4 μ m, methyl silicone, 40°C for 6 min then 5°C/min to 220°C. Sampling by dynamic solvent effect, 15 ml/min for: (A) 20 min, (B) 10 min, 22–23°C, *n*-hexane as solvent. Detection by FID, sensitivity 4×10^{-11} A/mV, full-scale deflection. Peak numbers correspond to identities in Table 1.

TABLE 1. VOLATILE COMPOUNDS FROM ANAL GLAND SECRETION OF AARDWOLF IDENTIFIED BY HIGH-RESOLUTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY^a

Peak number	Compound
1	Methylpropanoic acid
2	Butanoic acid
3	Hexan-1-ol
4	2-Methylbutanoic acid
5	Pentanoic acid
6	2-Methylpentanoic acid
7	Hexanoic acid
8	Ethyl heptanoate
9	Heptanoic acid
10	Ethyl octanoate
11	Octanoic acid
12	Indole
13	Butyl heptanoate
14	Propyl octanoate
15	Ethyl nonanoate
16	Hexyl hexanoate
17	Pentyl heptanoate
18	Butyl octanoate
19	Ethyl decanoate
20	Heptyl hexanoate
21	Hexyl heptanoate
22	Pentyl octanoate
23	Butyl nonanoate
24	Ethyl undecanoate
25	Heptyl heptanoate
26	Hexyl octanoate
27	Pentyl nonanoate
28	Butyl decanoate
29	Heptyl octanoate
30	Hexyl nonanoate
31	Pentyl decanoate
32	Octyl octanoate
33	Heptyl nonanoate

^aPeak numbers correspond to Figure 1.

in the anal gland secretions of hyenids is uncertain; Wheeler et al. (1975) reported only 5-thiomethylpentane-2,3-dione and one unidentified volatile component from *Hyaena hyaena*, to which Wheeler (1977) added a macrocyclic lactone. Examination of chromatograms from extracts of brown hyena (*H. brunnea*) pastings (Mills et al., 1980) reveals no peaks with the shape expected

for free fatty acids on an apolar phase, although fatty acids have been found in the headspace of brown hyena pastings (Apps, unpublished)

The occurrence of a complex series of short- and medium-chain esters is unusual in mammalian scent secretions. C₁₆ and C₁₈ acetates have been reported from bank vole (*Clethrionomys glareolus*) urine (Brinck and Hoffmeyer, 1984), and long-chain esters and squalene make up 96% of the scent mark of the saddle back tamarin (*Saquinus fuscicollis*) (Yarger et al., 1977). Esters similar to those from the aardwolf have been reported only from rabbit (*Oryctolagus cuniculus*) feces (Goodrich et al., 1981) and the anal sac of the wolf (*Canis lupus*) (Raymer et al., 1985). Esters may well be more widespread; sample pretreatments involving acidic or basic extractions (e.g., Maurer et al., 1979) are likely to lead to their loss by hydrolysis. No sulfur compounds were identified, although the metallic component of the odor suggests that they may be present. They certainly do not occur in the high concentration (1%) reported for 5-thio-methylpentane-2,3-dione in the anal gland secretion of *H. hyaena* (Wheeler et al., 1975). Indole occurs in the anal gland secretion of *Mustela* spp., but not in other Mustelidae (Brinck et al., 1983), and is a minor component in the anal sac of the wolf (*Canis lupus*) (Raymer et al., 1985). It has also been found in the anal gland secretions of the porcupine (*Hystrix africae australis*) (Apps et al., unpublished) and the guinea pig (*Cavia aperea* and *C. porcellus*) (Wellington et al., 1979).

Gosling (1982) has suggested that scent marks demarcating a territory should have an odor unique to their producer. Individual differences in mammalian odors are possibly based on differences in the relative concentrations of components of complex mixtures (Gorman, 1980). If both these hypotheses are true in the case of the aardwolf, the series of esters in its anal gland secretion appears to have the properties that would be expected to be appropriate to this role.

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DEFENSIVE SECRETION OF THE CARABID BEETLE
*Pasimachus subsulcatus*¹

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Abstract—The defensive secretion of the carabid beetle *Pasimachus subsulcatus* is a concentrated solution (up to 90%) of carboxylic acids, amounting to about 1% of body mass. It contains three major components (methacrylic, tiglic, and angelic acids) and four minor components (isobutyric, 2-methylbutyric, isovaleric, and senecioic acids). In the single population of this large flightless beetle that was examined, the relative ratio of acidic components was remarkably constant from individual to individual.

Key Words—Defensive secretion, aliphatic acids, beetle, Coleoptera, Carabidae, *Pasimachus subsulcatus*, carboxylic acids.

INTRODUCTION

The beetles of the family Carabidae possess a pair of dischargeable defensive glands, so-called pygidial glands, that open near the tip of the abdomen. Much is known about the chemistry of the secretion of these glands (Blum, 1981). The genus *Pasimachus* has itself been the subject of earlier work, which led to characterization of methacrylic acid as major component in the glands of one species (*P. elongatus*) and tentative characterization of this compound in the glands of two others (*P. duplicatus*, *P. californicus*) (McCollough, 1969, 1972).

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We report here on the secretory chemistry of *P. subsulcatus*, a large flightless species from the southeastern United States. We studied individuals from a single population of this beetle. Our purpose was to characterize as fully as possible the composition of the secretion and to determine the extent of its chemical variability within the population.

METHODS AND MATERIALS

Biological Procedures. The beetles were taken in pit traps in a 50-hectare plot (upland sandhill habitat) of the ecological research area of the University of South Florida (Tampa, Hillsborough County). To test for the beetle's responsiveness to disturbance, individuals were manipulated or held by hand while one or more of their legs were pinched in forceps. Others were stimulated while being held on indicator paper (filter paper soaked in alkaline phenolphthalein solution). Ejections became visible on such paper by the change in indicator color from red to white.

A total of 29 beetles (17 males, 12 females) were individually killed by slow freezing (which fails to induce them to discharge) and dissected under saline. The two glands of each were excised and ruptured in a small vial under dichloromethane. In 11 of these individuals (nine males, two females), the glands were weighed before and after rupturing, thus providing a measure of total mass of secretion per beetle. Two additional beetles, both males, were similarly dissected, but their two glands were extracted separately.

Chemical Analysis. In an exploratory procedure, portions of the dichloromethane extracts were subjected to gas chromatographic (GC) analysis both before and after treatment with diazomethane. Diazomethane was generated by the treatment of Diazald (*N*-methyl-*N*-nitroso-*p*-toluene sulfonamide; Aldrich Chemical Co.) in diethoxyethane with a 1:1 mixture of aqueous potassium hydroxide (10.7 M) and ethanol (95%).

Both the acids and the methyl esters produced by diazomethane treatment were analyzed by gas chromatography, gas chromatography-mass spectrometry (GC-MS), and proton nuclear magnetic resonance spectroscopy ($[^1\text{H}]\text{NMR}$).

GC was performed on a Varian series 2100 Aerograph instrument equipped with glass columns and a flame ionization detector using nitrogen as the carrier gas. A column of 5% Carbowax 20 M on 100/200 Supelcoport, 3.8 m \times 4 mm (50–150°C at 10°/min) served for separation of free acids and methyl esters; a column of GP 10% SP-1200/1% H₃PO₄ on 80/100 Chromsorb W AW, 3.5 m \times 4 mm (isothermal at 150°C) was also used for separating free acids. Capillary GC was carried out on a Shimadzu Mini-2 gas chromatograph equipped with a flame ionization detector, split injector, a 0.25- μm coated Carbowax 20 M fused silica column, 15 m \times 0.25 mm (100–150°C at 6°/min) and a Shi-

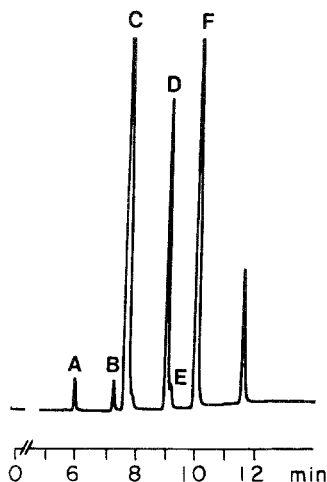


FIG. 1. Gas chromatogram of *P. subsulcatus* defensive secretion. Unlettered peak is internal standard (heptanoic acid). Details in text.

madzu C-R1A integrator. Helium was used as the carrier gas. Since two acids overlapped with this system, later studies used a Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector, a splitless on-column injector, and a 5.0- μm coated methyl silicone fused silica column, 15 m \times 0.53 mm (35°C for 0.5 min, 25°/min from 35°C to 50°C, and 6°/min from 50°C to 100°C). A Hewlett Packard 3392A integrator was used. Hydrogen was used as carrier gas with nitrogen as the makeup gas.

Quantification was done using heptanoic acid as internal standard. For each determination an average was taken, based on two replicate runs (replicate values usually differed by less than 2%). Detector responses were calibrated using authentic methacrylic acid as a standard for peaks A, B, and C, while tiglic acid was used as a standard for peaks D, E, and F (Figure 1).

Packed column GC-MS was performed on a Finnigan 3300 instrument interfaced with a Systems Industries 150 data system. Capillary GC-MS was performed on a Hewlett Packard 5987A mass spectrometer and a Finnigan 4500 instrument.

For preparative GC purposes, a 9:1 effluent splitter was attached to the Varian 2100 gas chromatograph. The effluent was collected in a 2-mm-ID tube cooled by a Dry Ice-acetone bath. The tubes were rinsed with the appropriate solvent for NMR analysis.

Proton NMR spectra were obtained on a Bruker WM-300 spectrometer using deuteriochloroform as the solvent.

Authentic samples of methacrylic, tiglic, isobutyric, isovaleric, and sene-

coic acids were obtained from commercial sources; angelic acid was prepared through isomerization of tiglic acid (Buckels and Mock, 1950); 2-methylbutyric acid was prepared by Jones oxidation of 2-methylbutanol.

RESULTS

Defensive Behavior and Gland Structure. When first taken from the pit traps, the beetles tended to discharge readily in response to manipulation, ejecting their secretion forcibly as a spray, often to a distance of several centimeters. After laboratory confinement they could usually be handled gently without being caused to discharge, although pinching of legs or persistent rough handling of the body almost invariably elicited ejections. Discharges were always accompanied by a pungent odor. Vapor from the secretion was irritating to the eyes, and the fluid itself, if it contacted abraded skin, induced immediate pain.

As evidenced by the patterns left on indicator paper, glandular discharges were sometimes unilateral: if stimulation was restricted to one side, the beetles usually discharged from that side only. Glandular emissions sometimes took the form of liquid outflowings rather than forcible ejections. Following stimulation, fluid would suddenly emanate from the gland openings and spread over the beetle's rear and forward along its flanks. In beetles placed on indicator paper, such discharges manifested themselves by the appearance of a zone of gradual discoloration around the beetle's periphery, as vapors emanated from its body (Figure 2, left).

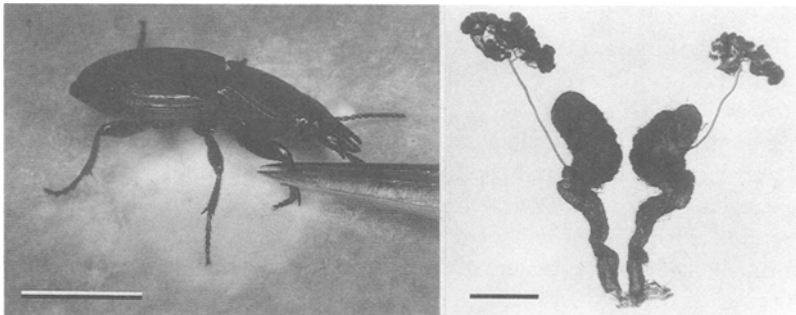


FIG. 2. (Left) *Pasimachus subsulcatus* being held by right foreleg with forceps on sheet of red indicator paper (filter paper soaked in alkaline phenolphthalein solution). The beetle has discharged secretion, which has oozed from the glands and spread forward over parts of its body. Vapor emanating from the acidic secretion has induced a zone of discoloration on the paper. (Right) Defensive glands of *P. subsulcatus*. Each consists of a cluster of glandular tissue, joined to the saclike reservoir by a narrow efferent tube. The two reservoirs (here shown in partly replete condition) lead to the outside (bottom of photo) by two narrowed ducts. (Reference bars: left, 1 cm; right, 1 mm).

P. subsulcatus has an exceedingly tough integument and a powerful set of mandibles, attributes that must themselves contribute to defense. Beetles that we handled frequently tried to inflict bites.

The glands (Figure 2, right) conform closely in gross structure to those described for *P. elongatus* by Forsyth (1972). Each consists of a racemose cluster of glandular tissue, with an efferent duct leading to a capacious reservoir. From the weighings of glands before and after rupturing, the following values were obtained for mass of secretion per pair of glands of beetle: males ($N = 9$), 4.5 ± 0.6 mg ($X \pm SE$) (range: 2.4–8.9 mg); females ($N = 2$), 1.0 and 6.1. Mass of whole beetles ($X \pm SE$) was as follows: males ($N = 15$), 391 ± 13 mg (range: 318–499 mg); females ($N = 11$), 446 ± 20 mg (range: 356–574 mg). The secretion therefore amounted, on average, to about 1% of body mass.

Composition of Secretion. GC comparison of defensive secretion before and after treatment with diazomethane showed all components to be methylated by this reagent, indicating that they are carboxylic acids.

Packed column GC separated two major acidic components and two minor ones. Subsequent analysis of extracts by capillary GC and GC-MS showed the presence of three additional minor components, giving a total of seven acidic compounds (Figure 1), two of which were unresolved under peak B.

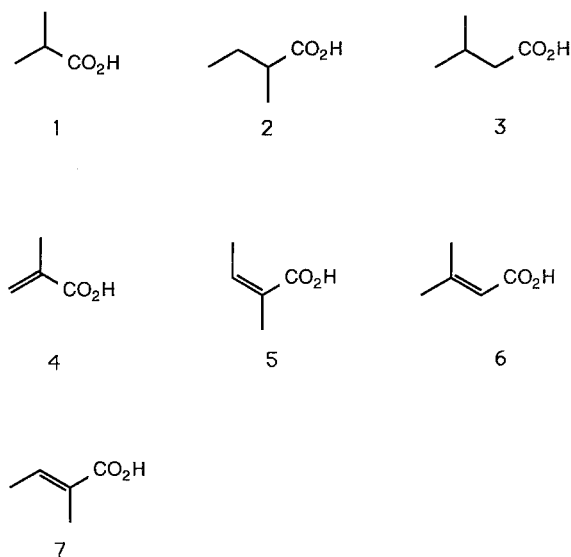
By matching GC peak retention times with retention times of authentic samples, after consideration of the GC-MS and [^1H]NMR data, the following components were identified.

Isobutyric Acid. Peak A cochromatographed with authentic isobutyric acid (1, Scheme 1). Electron impact (EI) GC-MS showed the molecular ion to be at $m/z = 88$ (CI-MS: 89) and gave a fragmentation pattern close to that reported in the literature (Heller and Milne, 1978) and identical to that of authentic isobutyric acid. The [^1H]NMR spectrum of peak A matched the spectrum of an authentic sample of isobutyric acid, as well as that previously reported (Pouchert and Campbell, 1974).

2-Methylbutyric acid and Isovaleric Acid. Peak B cochromatographed with both 2-methylbutyric (2) and isovaleric acid (3). The EI GC-MS spectrum was consistent with that expected for the superposition of the reported spectra of these acids (Heller and Milne, 1978). Subsequent analysis on another capillary GC system resolved the two acids and the retention times were matched to the appropriate authentic samples. The ratio of 2-methylbutyric to isovaleric acid is approximately 4:1.

Methacrylic Acid. Peak C cochromatographed with authentic methacrylic acid (4). EI GC-MS showed a molecular ion of $m/z = 86$ (CI-MS: 87) and gave a fragmentation pattern similar to that reported in the literature (Heller and Milne, 1978) and identical to an authentic sample. The [^1H]NMR spectrum matched that previously reported (Pouchert and Campbell, 1974).

Angelic Acid and Senecioic Acid. Peaks D and E cochromatographed with authentic samples of angelic (5) and senecioic (6) acids, respectively. Due to



SCHEME 1.

incomplete resolution of compounds **5** and **6** during preparative GC, a $[^1\text{H}]$ NMR spectrum of the mixture of acids was obtained. Nevertheless, the peaks observed could be matched with peaks in the individual $[^1\text{H}]$ NMR spectra of authentic samples of angelic and senecioic acids. Peak integration indicated the ratio of angelic to senecioic acid to be approximately 3:1. Capillary GC-MS gave molecular ions of $m/z = 100$ for both acids. The fragmentation patterns of peaks D and E matched those of authentic samples of angelic acid and senecioic acid respectively.

Tiglic acid. Peak F cochromatographed with authentic tiglic acid (**7**). EI GC-MS gave a molecular ion at $m/z = 100$ (CI-MS: 101), and a fragmentation pattern similar to that reported for 2-methyl-2-butenoic acid (of unspecified stereochemistry, Heller and Milne, 1978) and identical to that of authentic tiglic acid. The $[^1\text{H}]$ NMR spectrum matched that reported in the literature (Pouchert and Campbell, 1974).

Relative and absolute amounts of these components, as obtained from paired-gland extracts of individual beetles, were calculated from the capillary column GC analytical data, as described under Methods and Materials. The results are summarized in Figure 3, in which analyses for males (17), females (11), and one unique female are plotted separately.

Based on the values for total mass of secretion per beetle and total fatty acid per beetle, the following values were calculated for percent total fatty acid in secretion: males ($N = 9$), $67 \pm 4\%$ ($X \pm \text{SE}$) (range 46–87%); females ($N = 2$), 57 and 68%. The data for the two males whose four glands were individually extracted are shown in Figure 4. Ratio of components was in line with

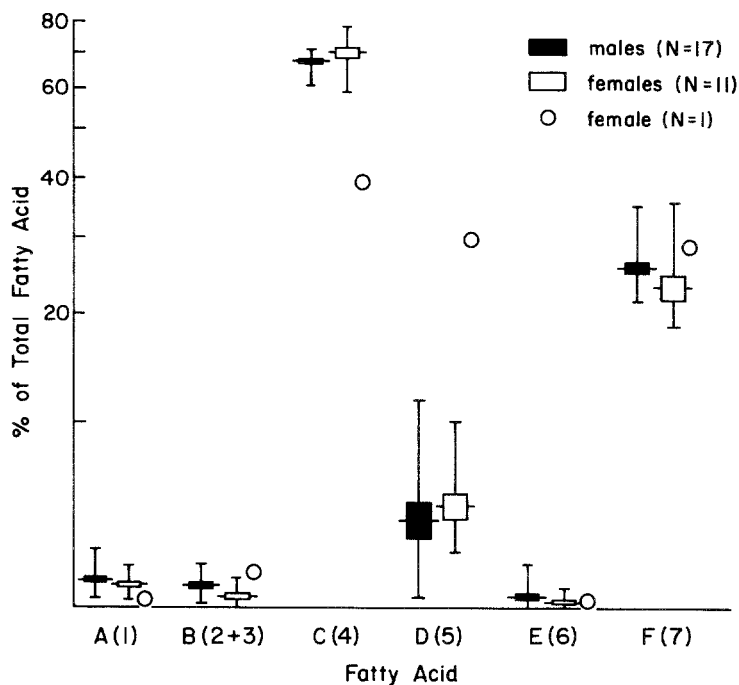


FIG. 3. Relative concentration of fatty acids in defensive secretion of male and female *P. subulcatus*. The one female whose composition was discrepant is plotted separately. Letters and numbers refer respectively to chromatographic peaks (Figure 1) and chemical structures figured in text. Mean \pm SE, and ranges are shown.

Gland	% Total Fatty Acid in Secretion	Relative % of Fatty Acids					
		1	2 + 3	4	5	6	7
1A	80.0	0.6	0.7	65.1	1.8	0.2	31.5
1B	90.1	0.4	0.7	59.1	3.1	0.3	36.4
2A	79.8	0.8	0.9	63.6	3.6	0.2	30.9
2B	—	0.9	1.4	61.1	3.2	0.1	33.3

FIG. 4. Composition of the two glands (A and B) of two individual male beetles (1 and 2). Numbers above vertical columns correspond to chemical structures in text. Gland 2B lost some of its contents through leakage following weighing, precluding determination of percent total acid content.

the norm for the beetles (Figure 3) and comparable for the two glands of each pair.

DISCUSSION

The first isolation of a chemical component from the defensive secretion of an arthropod was that of formic acid from ants (Wray, 1670). Carboxylic acids have since been isolated in substantial diversity from defensive glands of other arthropods, including whip scorpions, millipedes, hemipterans, lepidopterans, and beetles (Blum, 1981). Within the family Carabidae itself, carboxylic acids have been reported from a diversity of genera. All components from *P. subsulcatus* are known from other carabids (Blum, 1981; Kanehisa and Kawazu, 1982), and the combination of methacrylic and tiglic acid as major constituents, first reported by Schildknecht and Weis (1962), is not uncommon (Kanehisa and Kawazu, 1982).

The constancy in composition of the secretion from individual to individual in our *P. subsulcatus* population was striking. Only one of 31 individuals showed a ratio of components notably different from the norm. Ratios were comparable for males and females, and for the two glands of a pair.

One wonders what to make of the one female whose secretory composition was discrepant. In the absence of data on geographical variability of the secretion, it is impossible to tell whether the aberrant female is a local variant or an immigrant from another site. Given the flightlessness of *Pasimachus*, and its consequent potential tendency toward regional genetic stabilization, it might be interesting to see whether secretory composition could serve as an indicator for study of the population dynamics of the species. We cannot categorically rule out the possibility that the aberrant female was taxonomically misidentified. It seems unlikely, however, that the specimen belonged to the congeneric *P. strenuus*, which also occurs in the area, since in several Florida populations of this species that we examined the secretion consistently contained only minimal amounts (<0.5%) of tiglic acid (S. Schultze, M. Deyrup, J. Meinwald, T. Eisner, unpublished).

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DISRUPTION OF WEB STRUCTURE AND PREDATORY BEHAVIOR OF A SPIDER BY PLANT-DERIVED CHEMICAL DEFENSES OF AN APOSEMATIC APHID

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Abstract—Two toxic and bitter-tasting cardenolides (cardiac-active steroids) were sequestered by the brightly colored oleander aphid, *Aphis nerii* B. de F., from the neotropical milkweed host plant *Asclepias curassavica* L. After feeding on milkweed-reared aphids, the orb-web spider *Zygiella x-notata* (Clerck) built severely disrupted webs and attacked fewer nontoxic, control aphids, whereas the webs of spiders fed only nontoxic aphids remained intact. The regularity and size of the prey-trapping area of webs were reduced significantly in proportion to the amount of toxic aphids eaten. The effects of toxic aphids on spider web structure were mimicked by feeding spiders the bitter-tasting cardenolide digitoxin, a cardenolide with similar steroidal structure and pharmacological activity to the two aphid cardenolides. These results show that the well-known effects of psychoactive drugs on spider web structure are more than interesting behavioral assays of drug activity. Similar effects, produced by plant-derived chemicals in the spider's aphid prey, are relevant to the ecology and evolution of interactions between prey defense and predator foraging.

Key Words—Spider web structure, *Zygiella x-notata*, Araneae, predator foraging, aposematic aphid prey, *Aphis nerii*, Homoptera, Aphididae, chemical defense, cardenolides, *Asclepias*, milkweed.

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INTRODUCTION

Intriguing experiments with the effects of psychoactive drugs on spider web structure have shown that amphetamines, mescaline, psilocybin, LSD, and caffeine fed to spiders can induce dramatic changes in their webs (Witt and Reed, 1965; Witt et al., 1968; Jackson, 1974). However, such imaginative assays of drug activity have little relevance to the primary use of webs in prey capture by spiders, since the drugs do not occur in the predominantly insect prey of spiders (Blum, 1981).

At the time that drugs were first found to affect spider webs, plant-derived chemical toxins were also identified in some brightly colored insect herbivores feeding on milkweeds (Parsons, 1965; Reichstein, 1967; Reichstein et al., 1968; Brower et al., 1968; Brower, 1969; Rothschild et al., 1970). These toxins proved to be bitter-tasting cardiac-active steroids, or cardenolides, that caused bird predators to reject the insects on sight, thus confirming the hypothesized aposematism of their bright coloration. Many insect species are now known either to sequester or synthesize a wide range of toxic defensive chemicals (Blum, 1981).

Chemical disruption of spider web structure and the demonstration of chemical defenses in insects suggest that chemically defended prey of spiders could disrupt spider web structure much like some psychoactive drugs. Thus I wished to determine whether known insect toxins, such as cardenolides, in aposematic spider prey could disrupt the web structure of an orb-web spider and whether spider predatory behavior might be influenced by chemically defended prey.

Orb spiders site their webs in areas of high prey density (Turnbull, 1964; Gillespie, 1981; Janetos, 1982, 1986; Olive, 1982; Riechert and Luczak, 1982; Riechert and Gillespie, 1986) to intercept a wide diversity of different kinds of mobile prey. Some of these prey will have defensive chemicals that may make the spider release them unharmed (Brown, 1984; Vasconcellos-Neto and Lewinsohn, 1984) or, if the spider eats them, may disrupt its web-building physiology and behavior, much like the psychoactive drugs. Consequently, spiders should include prey defenses, such as toxic chemicals, when assessing prey profitability (Stephens and Krebs, 1986).

Since aphids make up a significant proportion of the diet of the araneid spider *Zygiella x-notata* (Nyffeler and Benz, 1981; personal observations), and the bright yellow and black, aposematic oleander aphid *Aphis nerii* B. de F. sequesters toxic cardenolides from the milkweed food plant *Asclepias curassavica* L. (Rothschild et al., 1970; Malcolm, 1981, 1986), I investigated the influence of *A. nerii* on the web structure and predatory behavior of *Z. x-notata*. The three trophic level interaction among *A. curassavica*, *A. nerii*, and *Z. x-notata* is often associated with houses and gardens, and as such has been dis-

tributed throughout the warmer parts of the world, from the Mediterranean to Caribbean islands, Chile, Uruguay, Argentina and on both the west and east coasts of North America (Woodson, 1954; Gertsch, 1964; Levi, 1974).

METHODS AND MATERIALS

Spiders were collected in late March from a single egg sac on a stone wall near Oxford. At first, as is normal, each *Z. x-notata* spiderling built an entire orb web without the spiral-free sector that is characteristic of the species (Witt et al., 1968). When the spiders began to build their characteristic webs, after the second or third molt, 13 female spiderlings were induced to build webs in 120 × 120 mm balsa wood frames (Figure 1), each suspended in a 23 × 23 × 10 cm clear plastic box at 15°C, in a constant environment room with 12 hr light.

Web structure was compared for spiders fed toxic *A. nerii*, palatable green pea aphids, *Acyrtosiphon pisum* (Harris), and two commercially available cardenolide standards. For these experiments *A. nerii* was reared on cardenolide-rich *A. curassavica*, and *A. pisum* was reared from broad beans, *Vicia faba* L.

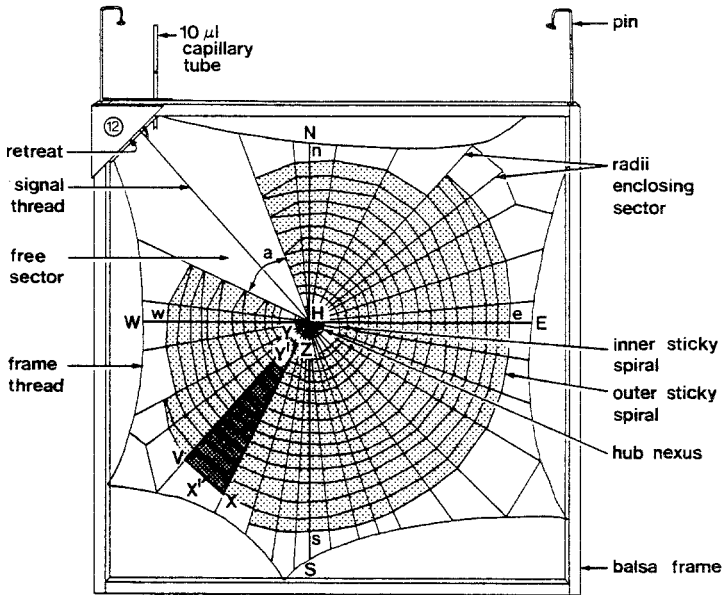


FIG. 1. The web of a *Z. x-notata* spiderling in its balsa frame and the coordinates used to calculate the sticky spirals area (stippled, sum of $VXYZ$), the length of the sticky spiral thread that makes this area, and the average mesh size of the sticky net.

cv *aquadulce*. The *A. curassavica* host plants were grown from plants of unknown origin, via the botanical gardens at Kew and Oxford (the same strain of plants described by Rothschild et al., 1975), and *A. nerii* was collected originally by Miriam Rothschild on *Nerium oleander* in Israel.

There were four feeding treatments involving the 13 spiders: (1) Four experimental spiders were fed once a week with one *A. nerii* followed after 30 min by one green pea aphid, plus a constant supply of 50% sucrose solution; (2) three control spiders were fed one pea aphid weekly, plus a constant supply of 50% sucrose solution; (3) three spiders were fed one pea aphid weekly plus the cardenolide digitoxin, constantly available in 50% sucrose solution (0.014 mg/ml, the maximum solubility of digitoxin in aqueous solution, Wright, 1960); and (4) three spiders were fed one pea aphid weekly plus the polar cardenolide ouabain, constantly available in 50% sucrose solution (1.0 mg/ml). Thus treatments 1 and 2 examined the influence of aphid prey on spider predation and treatments 3 and 4 examined the influence of cardenolide standards on spider predation.

Throughout the experiments, sucrose solution, with or without added cardenolide, was held in a 10 μ l capillary tube by each spider's retreat. Aphids were placed with forceps on the web within 1 cm of the hub and left until discarded by the spider. Spiders were allowed to build repeated webs unmolested and webs were not artificially damaged or destroyed to induce new web construction. These feeding treatments were run for 70 days, during which time spiders were always in their retreat, with one leg on the signal thread (Figure 1) before they were fed.

The presence or absence of a spider response to an aphid (moving from the retreat to touch an aphid) during the first 30 min of a prey presentation was recorded. The time to attack a presented aphid and the time spent on the web in response to an aphid presentation were measured in seconds during this period. Ultimate aphid acceptance (aphid partially or completely eaten) or rejection (intact aphid discarded), which took from hours to days, was also recorded.

The feeding regimes controlled for possible developmental responses of the spiders to their prey (Murdoch, 1971) since the spiders weighed a mean of 1.01 mg (± 0.19 SE) at the start of the experiment and only increased to a mean weight of 1.37 mg (± 0.17 SE), 70 days later. Only one control spider molted during the experiment. Aphids given to spiders were matched for size, *A. nerii* weighing a mean of 0.61 mg (± 0.03 SE) and *A. pisum* 0.65 mg (± 0.09 SE). The wet weights of aphid prey were determined immediately before being fed to a spider and after they were discarded by the spider. Prey weights were approximately 50% of spider weight since this prey size is in the size range most acceptable to spiders (Nentwig and Wissel, 1986).

Experimental Design. In these experiments the replicated experimental units were, (1) direct behavioral responses, and (2) indirect web structure

responses, of spiders to each food treatment. This design precluded temporal pseudoreplication (Hurlbert, 1984) because each replicate (both direct and indirect responses) was an independent response to each feeding treatment. Treatments were interspersed among spiders but not within spiders. Each replicate also had controls for both temporal (the control treatment) and response variation (the web of each spider before treatment commenced). An experimental design with individual spiders as once-used replicates would not have included responses of successive web structure with time, web construction frequencies, dose dependency, or web structure recovery—all aspects considered important to the experiment. Furthermore, the experimental design minimized disturbance to the spiders and eliminated desertion of their balsa web frame.

Cardenolide Analysis. Since Rothschild et al. (1970) found two cardenolides in *A. nerii* from *A. curassavica*, both aphid prey species were assayed for the presence of cardenolides to ensure that spiders received cardenolide in *A. nerii* but not in *A. pisum*. Freeze-dried aphids were homogenized under nitrogen with chloroform-methanol (2:1, v/v) and centrifuged. Concentrated filtrates were eluted with the cardenolide standard digitoxin on 0.2 mm silica gel-60 thin-layer chromatograms at 15°C in the solvent system dichloromethane-methanol-formamide (80:10:1). Cardenolides were detected as blue spots on the developed TLC plates sprayed with alkaline 2,2', 4,4'-tetranitrodiphenyl. The spectrophotometric method of Brower et al. (1975) was used to estimate the cardenolide concentrations of aphids.

The host plants *A. curassavica* and *V. faba* were also assayed qualitatively for the presence of cardenolides. Freeze-dried leaves were macerated and extracted in 65% aqueous methanol and partitioned with hexane. The methanol fraction was extracted three times with chloroform and then extracted three times with chloroform-ethanol (3:2 v/v). The chloroform and chloroform-ethanol extracts were washed with Na₂SO₄ solution and dried over anhydrous Na₂SO₄ and then filtered through Whatman No. 1 filter paper and reduced under vacuum to approximately 1 ml. These concentrated extracts were then eluted in the same TLC system as the aphid extracts.

Web Structure Measurement. Web coordinates were measured (Figure 1) from projected 35-mm negatives, photographed in a light box (after Witt et al., 1968), to give: (1) the sticky spiral area as the sum of the triangles bounded by all radii and the outer sticky spiral, minus the triangle from the hub to the inner sticky spiral. Thus the sticky area = sum of $VXZY$ except the free sector a , where $VXZY = VXH - YZH$ when $VXH = (X'H/m \times VX/m)/2$ and $YZH = (Y'H/m \times YX/m)/2$, and m = magnification of the projected image. (2) Length of the sticky thread, $L = b[n\pi(h + o)]$, where $b = (360 - a)/360$, a = free sector angle, n = mean spiral number, h = lengths of radii at hub ($Y'H$) summed/number of radii - 1, and o = lengths of radii at outer spiral ($X'H$) summed/number of radii - 1. (3) Mesh size = sticky area/number of radii \times

mean spiral number, where mean spiral number = sum of spiral number in each sector/number of radii - 1. These calculations were made using SPSS (Nie et al., 1975).

RESULTS

Aphid and Plant Cardenolides. Two cardenolides were found in prey *A. nerii* but not in *A. pisum*. The solvent mobilities of these cardenolides, relative to digitoxin, were 1.31 (mean \pm 0.08 SD, $N = 7$) and 1.73 (mean \pm 0.21 SD, $N = 7$), which suggests that they are the cardenolides proceroside and calotropin, respectively, found previously in *A. nerii* by Rothschild et al. (1970). The concentration of cardenolides in *A. nerii* from *A. curassavica* was 246 $\mu\text{g}/0.1$ g dry aphid, with an average of 0.5 μg cardenolide in each aphid.

No cardenolides were found in extracts of *V. faba*, the host plant of *A. pisum*. However, 20 cardenolides were identified in TLC separations of *A. curassavica* extracts. Two of these spots had mean mobilities relative to digitoxin of 1.21 (\pm 0.05 SD, $N = 5$) and 1.66 (\pm 0.02 SD, $N = 5$) and did not differ significantly from the two aphid mean spot mobilities of 1.31 and 1.73 ($\chi^2 = 0.08$ and 0.14, respectively; both $df = 4$, and both $P = \text{NS}$ at 5%). Thus, although the mean mobilities of the two plant cardenolides were slightly less than those of the aphid, perhaps because the plant has more cardenolide material, the insignificance of this difference indicates that the aphid cardenolides were plant-derived.

Spider Predatory Behavior. Spiders attacked aphids by moving from their retreat rapidly down the signal thread to the hub and then along the radius on which an aphid was trapped. The aphid was usually bitten immediately and then either cut from the web and taken back to the retreat or left to die while the spider returned rapidly to its retreat. From 79 timed observations of the two aphid species (Table 1), there were no significant differences in the numbers of spider responses to aphids among the treatments ($\chi^2 = 5.76$, $df = 4$, $P = 0.22$). However, only 20% of *A. nerii* were ultimately accepted (killed and at least partially eaten) by experimental spiders, significantly fewer than the 85% of *A. pisum* accepted by all spiders ($\chi^2 = 35.37$, $df = 4$, $P = 0.001$).

The spiders in experimental treatment 1 attacked 76% of the *A. nerii* offered and 47% of the *A. pisum*, and spiders in groups 2, 3, and 4 (the control, digitoxin, and ouabain treatments) attacked 60%, 58%, and 83%, respectively, of their *A. pisum* (Table 1). Only 52% of attacked *A. nerii* were actually killed because 56% of attacks resulted in the spider withdrawing immediately after biting the aphid. These withdrawals may have been caused by cardenolides in *A. nerii* or by the fact that 64% of attacked *A. nerii* produced drops of strongly repellent supercooling cornicle secretion on attack (see Brown et al., 1969),

TABLE 1. OBSERVATIONS OVER 30 MIN OF PREDATORY BEHAVIOR OF SPIDERS TO TOXIC AND PALATABLE APHIDS IN FOUR FEEDING TREATMENTS WITH EXPERIMENTAL *A. nerii*, CONTROL, DIGITOXIN, AND OUABAIN SPIDERS

	Food treatment				
	Experimental		Control	Digitoxin	Ouabain
Aphid prey	<i>A. nerii</i>	<i>A. pisum</i>	<i>A. pisum</i>	<i>A. pisum</i>	<i>A. pisum</i>
<i>N</i>	25	15	15	12	12
Response (%)	76	47	60	58	83
Acceptance (%) ^a	20	67	87	92	100
Time to attack (sec)					
Mean	254	375	184	230	132
SE	80	183	109	122	83
<i>N</i>	19	6	9	7	10
Time on web (sec)					
Mean	347	198	184	501	271
SE	119	166	43	64	76
<i>N</i>	19	6	9	7	10

^aUltimate acceptance of aphids by the spiders, no time limit.

whereas only 6% of *A. pisum* in the same treatment did so. In contrast, 90% of attacked *A. pisum* were killed by spiders in the control, digitoxin, and ouabain groups because 47%, 64%, and 83%, respectively, of these aphids were held by the spiders after they were bitten.

A. nerii also appeared to inhibit attacks by the spiders on *A. pisum* in the experimental treatment because these spiders took twice as long to attack than control spiders and only responded to 47% of their palatable aphids, whereas they responded to 76% of the toxic *A. nerii* presented 30 min earlier (Table 1).

Although experimental and digitoxin spiders seemed to take longer to attack their aphid prey than ouabain and control spiders (Table 1), there were no significant differences among the feeding treatments (Kruskal-Wallis ranked ANOVA, $H_4 = 2.36$, $0.7 > P > 0.5$), since the times taken to attack aphids were highly variable (Table 1). However, at the 10% significance level, experimental and digitoxin spiders spent longer away from their retreats and on their webs in response to presentations of *A. nerii* and *A. pisum*, respectively (Table 1), than spiders responding to *A. pisum* in the experimental, control and ouabain treatments (Kruskal-Wallis ranked ANOVA, $H_4 = 8.53$, $0.10 > P > 0.05$). The experimental and digitoxin spiders often appeared disoriented after attacking an aphid and either wandered around the outer frame, or cleaned their legs and palps, or remained motionless with legs poised near an aphid, before returning to the retreat.

Prey Consumption. Overall prey consumption was examined by comparing the weight changes with time of prey aphids against the weight changes of similar-sized, intact, nonprey aphids. These nonprey aphids were held in similar containers to the prey aphids without access to host plants and hence controlled for weight loss by evaporation. A *t* test comparison between the regression slopes of *A. nerii* weight change with time showed no significant difference between prey and nonprey aphids ($t_{194} = -0.70$, $0.5 > P > 0.4$) because 80% of the prey aphids were intact and uneaten. Thus, although 52% of the *A. nerii* given to spiders were killed, only 20% were at least partially eaten.

In contrast, the spiders ate all of the body contents of the *A. pisum* they killed in each treatment, always discarding a crumpled, sucked-dry or chewed exoskeleton as the remains of their prey. The *t* test comparisons between regression slopes of prey and nonprey *A. pisum* wet weight loss with time in each treatment showed highly significant changes in prey aphids (digitoxin $t_{79} = -4.70$, $P < 0.001$; ouabain $t_{102} = -13.39$, $P < 0.001$; control $t_{90} = -10.57$, $P < 0.001$).

Changes in Web Structure with Time. Although the overall pattern of prey attack by experimental spiders showed rejection of most bitter-tasting *A. nerii* that they killed, each spider ate sufficient amounts of 20% of these aphids to produce dramatic changes in web structure.

An example of such a change is illustrated in Figure 2. The spider ate 68% of a 0.79 mg *A. nerii* placed in its web (Figure 2a) and built a seriously disrupted web two days later (Figure 2b). The next web built by the spider after a further four days showed some recovery, with an increase in trap area and thread regularity, although the web structure remained clearly disrupted (Figure 2c). These highly irregular webs are not the result of food limitation since at each prey presentation the spider completely consumed the palatable *A. pisum* presented, and hence had as much food as the group 2 control spiders which built no irregular webs.

The sticky area, length of the sticky thread that makes up the sticky area, and mesh size (see Figures 1 and 2) determine the web's trapping effectiveness (Rypstra, 1982; Murakami, 1983; Nentwig, 1983; Eberhard, 1986). Hence their changes with time were measured from photographs of the webs. Throughout the 70-day experiment the 13 spiders built 134 webs, distributed among the four treatments as shown in Table 2. Table 2 also shows the means, standard errors, and ranges of the calculated sticky areas, thread lengths, and mesh sizes of webs in each treatment.

The first webs built by spiders after being given an *A. nerii* had reduced sticky areas, reduced sticky thread lengths, and increased mesh sizes (Figure 3, Table 3). Each of these three measures of subsequently constructed webs showed a significant recovery with time following an encounter with *A. nerii*.

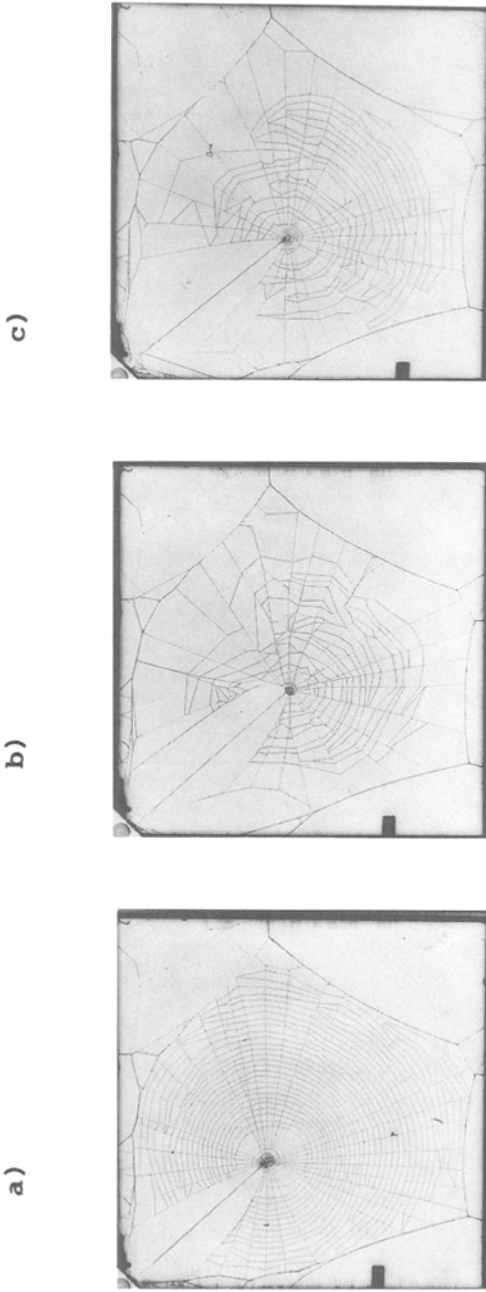


FIG. 2. Photographs of three consecutive webs built by a spider given the aphid *A. nerii*. (a) is "typical" of this spider individual and acts as a control for comparison with subsequently disrupted webs. Webs b and c were built two and six days, respectively, after the same spider ate 68% of an *A. nerii* placed in web a. Each web has the same outer frame thread, but the number of radii and the area of spiral sticky thread of webs b and c are much reduced. Accordingly, the length of sticky thread is much shorter and web mesh sizes are larger and more irregular. Web c shows some recovery of web structure with time since feeding on the toxic aphid. The number of radii have increased and the sticky thread area with smaller mesh sizes.

TABLE 2. SAMPLE SIZES, WEB FREQUENCIES, STICKY AREAS, STICKY THREAD LENGTHS, AND MESH SIZES OF WEBS CONSTRUCTED BY SPIDERS IN FOUR FEEDING TREATMENTS WITH *A. nerii*, *A. pisum* CONTROL, DIGITOXIN, AND OUABAIN

	Food treatment			
	<i>A. nerii</i>	Control	Digitoxin	Ouabain
Web frequencies (webs/70 days)				
Mean	8.25	11.33	13.00	9.33
SE	1.11	1.20	1.73	2.33
Range	6-11	9-13	10-16	7-14
Spider <i>N</i>	4	3	3	3
Sticky areas (mm ²)				
Mean	5231	4737	4657	5901
SE	192	199	185	294
Range	2926-7036	2638-7048	2219-7728	2969-7832
Web <i>N</i>	33	34	39	28
Sticky thread lengths (mm)				
Mean	2892	2857	2580	2747
SE	142	127	91	118
Range	1243-3938	1515-4482	1165-3650	1662-3627
Web <i>N</i>	29	32	37	28
Mesh sizes (mm ²)				
Mean	8.89	7.49	7.72	10.31
SE	0.29	0.20	0.14	0.35
Range	7.17-14.71	5.58-9.47	6.73-10.05	7.52-14.61
Web <i>N</i>	29	28	34	27

In contrast, the web structure of control spiders, fed only *A. pisum*, showed no significant changes (Figure 3, Table 3). Hence feeding encounters with *A. nerii* at first resulted in disrupted webs, but spiders recovered with time, and successive webs increased in regularity and size.

Reductions in the sticky areas and sticky thread lengths of webs, similar to those produced in the *A. nerii* treatment, were mimicked by giving spiders the cardenolide digitoxin, although mesh sizes were not significantly affected (Figure 3, Table 3). Where significant, these regressions have negative slopes because sticky web areas and thread lengths decreased significantly as the time exposed to digitoxin increased throughout the 70-day experiment. Like control spiders, the web structures of spiders in group 4, given the cardenolide ouabain, did not change significantly during the experiment.

Dose-Dependent Changes in Web Structure. Since 64% of *A. nerii* produced supercooling cornicle secretions in response to spider attacks, it is possible that web disruption may have been caused by the physical effects of this

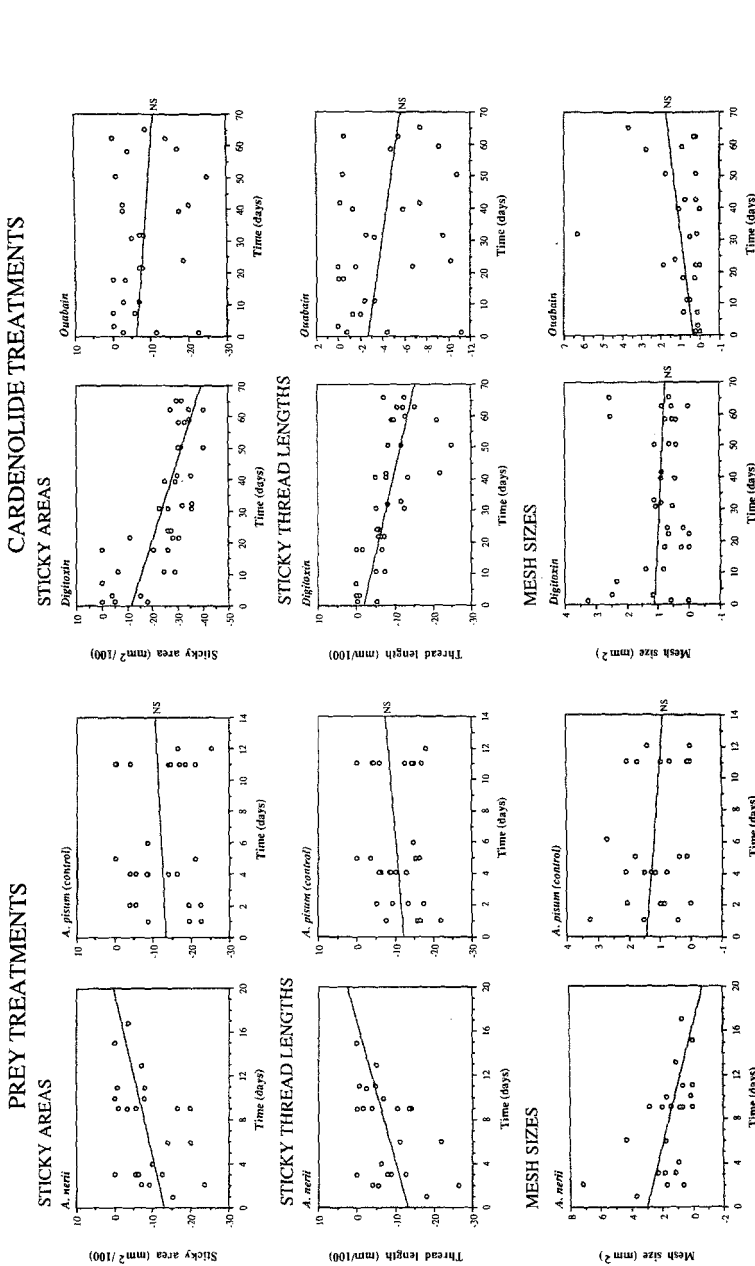


FIG. 3. Linear regressions of web measurements (sticky areas, sticky thread lengths, and mesh sizes) against time for spiders in the *A. neri*, control, digitoxin, and ouabain treatments. The regression data and their significance are given in Table 3 (NS = nonsignificant regression line). Significant regression slopes of sticky areas and sticky thread lengths increase with recovery in the *A. neri* treatment because webs return to their pretreatment size at the zero intercept with recovery in size since an aphid was given. The digitoxin treatment has negative slopes because webs were reduced in size with exposure to digitoxin during the entire 70-day experiment. Mesh sizes decreased with recovery in the *A. neri* treatment because unaffected mesh sizes are smaller and reflect the more effective trap of normal spider webs (see Figure 2).

cardenolide-free fluid (Malcolm, 1981) on the external leg receptors used by the spiders in web construction (Foelix, 1982). Thus in order to demonstrate that web structure is disrupted by the effects of aphid body contents containing cardenolides, the influence of the weight of *A. nerii* eaten with time (dose) on web structure was also examined.

The amount and rate of *A. nerii* eaten significantly reduced the sticky area of webs in a dose-dependent manner (Table 4), since the most *A. nerii* eaten in the shortest time produced the greatest reductions in web sticky area (Figure 4). The interaction was reversible, with less of an aphid eaten per unit time producing a smaller reduction in sticky area. In contrast, the rates of palatable aphid consumption in the control experiment had no effect on the sticky area of webs (Figure 4, Table 4).

Thus it is most likely that this dose-dependent web disruption was caused by aphid body contents containing cardenolides, rather than by the physical effects of hardened cornicle secretion.

TABLE 3. LINEAR REGRESSIONS OF FIGURE 3 DATA FOR CHANGES IN STICKY AREAS, STICKY THREAD LENGTHS, AND MESH SIZES OF WEBS BUILT BY SPIDERS IN EACH OF FOUR FOOD TREATMENTS AGAINST TIME^a

Food treatment	<i>N</i>	Intercept	Slope	<i>r</i> ²	<i>F</i>	<i>P</i> ^b
Sticky areas (mm ² /100)						
1. <i>A. nerii</i>	24	-13.19	0.67	0.20	5.51	0.028
2. <i>A. pisum</i> control	28	-13.40	0.19	0.01	0.27	NS
3. Digitoxin	39	-11.75	-0.39	0.53	41.81	0.0001
4. Ouabain	28	-6.26	-0.06	0.03	0.91	NS
Sticky thread lengths (mm/100)						
1. <i>A. nerii</i>	25	-13.56	0.79	0.23	6.87	0.015
2. <i>A. pisum</i> control	29	-12.32	0.33	0.05	1.39	NS
3. Digitoxin	37	-1.92	-0.19	0.47	31.13	0.0001
4. Ouabain	28	-2.76	-0.04	0.06	1.60	NS
Mesh sizes (mm ²)						
1. <i>A. nerii</i>	25	2.98	-0.19	0.28	9.07	0.006
2. <i>A. pisum</i> control	25	1.43	-0.04	0.04	0.87	NS
3. Digitoxin	34	1.10	-0.01	0.02	0.63	NS
4. Ouabain	28	0.33	0.02	0.09	2.66	NS

^aTime is measured in days since an aphid was given for spiders fed *A. nerii* and control spiders fed *A. pisum* and against days in the 70-day treatment for spiders given the cardenolides digitoxin, and ouabain. Web reduction is calculated by subtraction from the unaffected control webs built by each spider before its feeding treatment. The zero intercept represents this control web size (see Figure 3).

^bSignificant at < 0.05.

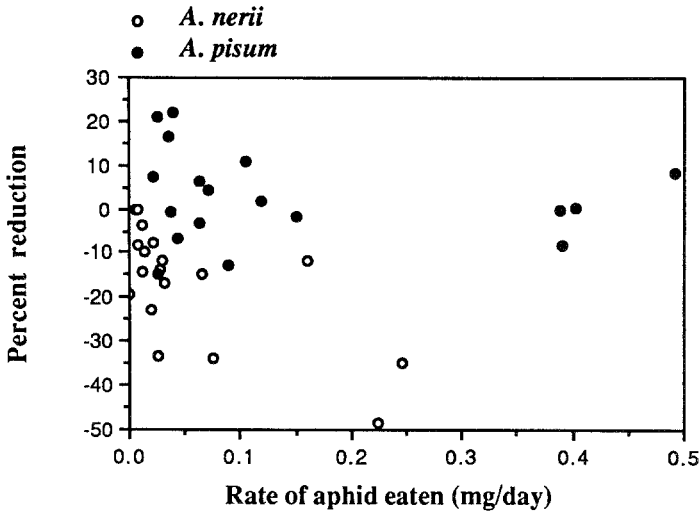


FIG. 4. Percent reduction in the sticky web areas of spiders fed either *A. nerii* or *A. pisum* versus the rate of aphid eaten. The data are transformed and analyzed in Table 4 and show that sticky web areas of spiders fed *A. pisum* do not change with rate of prey consumption but that the webs of spiders fed *A. nerii* show greater reductions in sticky areas with higher rates of aphid consumption.

TABLE 4. LINEAR REGRESSIONS OF FIGURE 4 DATA FOR PERCENT REDUCTION IN STICKY AREAS^a OF WEBS BUILT BY SPIDERS GIVEN EITHER DISTASTEFUL *A. nerii* OR PALATABLE *A. pisum* AS APHID PREY, AGAINST RATE OF APHID EATEN^b

Aphid prey	N	Intercept	Slope	r ²	F	P ^c
<i>A. nerii</i>	18	-0.104	-3.09	0.45	13.08	0.002
<i>A. pisum</i>	18	0.045	-0.30	0.03	0.41	NS

^a Arcsin transformed.

^b In mg/day log₁₀ transformed, corrected for the weight loss with time of control, nonprey aphids.

^c Significant at < 0.05.

DISCUSSION

Webs of spiders fed either *A. nerii* or the cardenolide digitoxin showed significant and similar kinds of disruption. This suggests that one or both of the two plant-derived aphid cardenolides caused the physiological and behavioral disruption of web structure. The highly polar ouabain produced no measurable

changes in web structure, and it is interesting that the aphid does not store any of the high-polarity cardenolides present in the host plant (Rothschild et al., 1970; Roeske et al., 1976; Malcolm, 1981). Ouabain is not a bitter-tasting cardenolide like digitoxin (Malcolm, 1981) and, owing to its high polarity, it is very slow to cross membranes (Wright, 1960; Duffey, 1977). Hence, ouabain may be excreted by the spider before any physiological effects are manifested. Ouabain has also been shown to have no effect on an ATP hydrolyzing enzyme in the central nervous system of *Z. x-notata* (Meyer, 1979), although in crustaceans it inhibits the stimulation of ATP (Lehninger, 1975). In contrast, bitter-tasting digitoxin is much less polar, like the cardenolides in *A. nerii*, and is readily absorbed across tissue and cell membranes (Wright, 1960; Duffey, 1977). Thus, orally administered digitoxin is expected to be considerably more toxic than ouabain to both invertebrate and vertebrate predators.

Construction of spider webs incurs time and energetic costs, associated with foraging for prey, that are balanced by the rewards of prey capture (Ford, 1977; Janetos, 1982; Olive, 1982; Rypstra, 1982; Eberhard, 1986). For these rewards to be maximized, prey capture efficiency is likely to be influenced by web structure. For example, Rypstra (1982) found the number of captured prey was maximized at a particular thread density, above which webs became increasingly visible and caught fewer prey (Craig, 1986). Prey size may be correlated with mesh size (Uetz et al., 1978), or mesh sizes may determine only the lower limit of captured prey size, because small insects can pass through the web mesh (Murakami, 1983). However, Nentwig (1983) found no correlation between prey size and mesh width. Instead, like Rypstra, he found that spiders with narrow-meshed webs caught more prey, but those with wide-meshed webs saved more web construction material. Hence the construction of webs by *Z. x-notata* with increased mesh sizes after feeding on *A. nerii* will decrease the capture efficiency of webs for small-sized prey. Feeding on *A. nerii* also resulted in fewer, smaller, and more irregular webs being built by *Z. x-notata*.

Changes in the web structure of *Z. x-notata*, induced by the chemical defense of an aphid prey, suggest that prey profitability and web site profitability for spiders is influenced by more than prey density or prey size (Janetos, 1982, 1986; Olive, 1982; Nentwig and Wissel, 1986; Riechert and Gillespie, 1986). The cardenolide-based chemical defenses of *A. nerii* clearly influence both the behavior and physiology of prey capture by *Z. x-notata*. The toxic aphid not only influenced predatory behavior indirectly, through altered web construction behavior, but also directly influenced predatory behavior, because spiders attacked fewer palatable *A. pisum* and took longer to attack them immediately after an encounter with toxic *A. nerii* than control spiders given only *A.*

pisum (Table 1). As Suter (1978) found with the orb spider *Cyclosa turbinata*, the spiders appeared unable to discriminate between the aphid prey species on the basis of web-borne vibrations alone.

The lengthened behavioral responses of *Z. x-notata* to aposematic *A. nerii* also meant that spiders were exposed for longer periods on their webs, displaying some evidence of disorientation, than spiders attacking only palatable prey. Thus, an additional cost of feeding on toxic prey may be increased exposure to natural enemies and abiotic mortality.

Web construction physiology was also influenced since spiders fed *A. nerii* built fewer webs (Table 2) than control spiders fed palatable aphids (Mann-Whitney $U_{3,4} = 1$, $P = 0.057$). Thus the mean web duration of spiders given *A. nerii* was also longer (8.95 days \pm 1.02 SE) than that of control spiders given *A. pisum* (6.33 days \pm 0.60 SE).

In order to maximize its foraging effectiveness, a spider must either move its web site, or stay and eat profitable prey, but not toxic prey, like *A. nerii*, trapped in its web. The first option has been documented for some web-building spiders, which remained longer at more profitable than less profitable trapping sites (Janetos, 1982; Olive, 1982; Riechert and Luczak, 1982); the probability of relocating a web and the distance moved both increased with decreasing site profitability. The alternative option, of staying at a web site and releasing trapped, unprofitable prey, has been recorded recently for a neotropical web-building spider that freed distasteful butterflies from their webs according to chemical cues (Brown, 1984; Vasconcellos-Neto and Lewinsohn, 1984). However, *Z. x-notata* was unable to inhibit completely its feeding response to *A. nerii*, which it continued to kill, and occasionally eat, throughout the experiment. Such feeding on chemically defended prey could reduce the numbers of subsequent prey caught, both indirectly by changing the trapping effectiveness of webs, and directly by inhibiting the predatory behavior of spiders. Prey-induced inhibition of spider predatory behavior is similar to the sedative effect of millipede chemical defenses against a wolf spider that Carrel and Eisner (1984) describe, but differs in that aphids are unlikely to escape a spider's web. Since *Z. x-notata* appears to attack automatically and kill both toxic and palatable prey (also, Malcolm, 1986), perhaps in response to similar frequencies of web-borne vibrations (Suter, 1978; Barth, 1982; Klärner and Barth, 1982; Masters, 1984a, b), prey choice may be determined primarily by web location within energetic and site availability constraints, rather than by discrimination among trapped prey items.

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SYNTHESIS AND PHEROMONAL ACTIVITY OF 6,10,13-
TRIMETHYL-1-TETRADECANOL FOR PREDATORY
STINK BUG, *Stiretrus anchorago* (HETEROPTERA:
PENTATOMIDAE)

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Abstract—Racemic 6,10,13-trimethyl-1-tetradecanol has been synthesized. Successive elaboration of the molecule using 5-methyl-2-hexanone as the carbon-terminal fragment gave the desired alcohol in a 4% overall yield over 13 steps. Neglecting stereochemistry, the product was shown to be identical to the alcohol previously identified from the sternal glands of male *Stiretrus anchorago*. It attracts both adult sexes and larvae of this insect in the field, confirming its postulated identity as an aggregation pheromone. The corresponding aldehyde is more than 10 times more concentrated in airborne-trapped samples (5–7% by gas chromatography) than in extracts of glandular setae (0.4–0.5%). This alcohol or its isovalerate ester has been found in other predaceous pentatomids, providing further evidence for pheromones in these species.

Key Words—Pheromone, attractant, *Perillus bioculatus*, *Oplomus severus*, *Mineus strigipes*, *Oplomus dichrous*, stink bugs, Heteroptera, Pentatomidae, 6,10,13-trimethyl-1-tetradecanol.

INTRODUCTION

For some years we have been engaged in exploration of the chemical communication of Heteroptera, particularly with respect to aggregation and/or sex pheromones. This subject has been reviewed recently (Aldrich, 1988).

Examination of five species of predatory asopine stink bugs (Aldrich et al.,

1986; Aldrich and Lusby, 1986) has shown the presence of 6,10,13-trimethyl-1-tetradecanol (in *Stiretrus anchorago* and *Mineus strigipes*), 6,10,13-trimethyl-1-tetradecyl isovalerate (in *Perillus bioculatus* and *Oplomus severus*), or a mixture of the two (in *Oplomus dichrous*) on setae associated with sternal glands present only in males. Identification was by spectrometric and degradative means (Aldrich et al., 1986) but the material was not synthesized.

Because of the potential use of a pheromone for population management of these beneficial insects, we undertook a synthesis of this alcohol. Since there are two asymmetric carbons in the molecule, there are four possible enantiomers. Optically pure materials are often not required, however, so we initially prepared the racemate by a sequential elaboration of 5-methyl-2-hexanone as summarized in Figure 1. The synthesis, comparison to the natural material, and field testing results are described here.

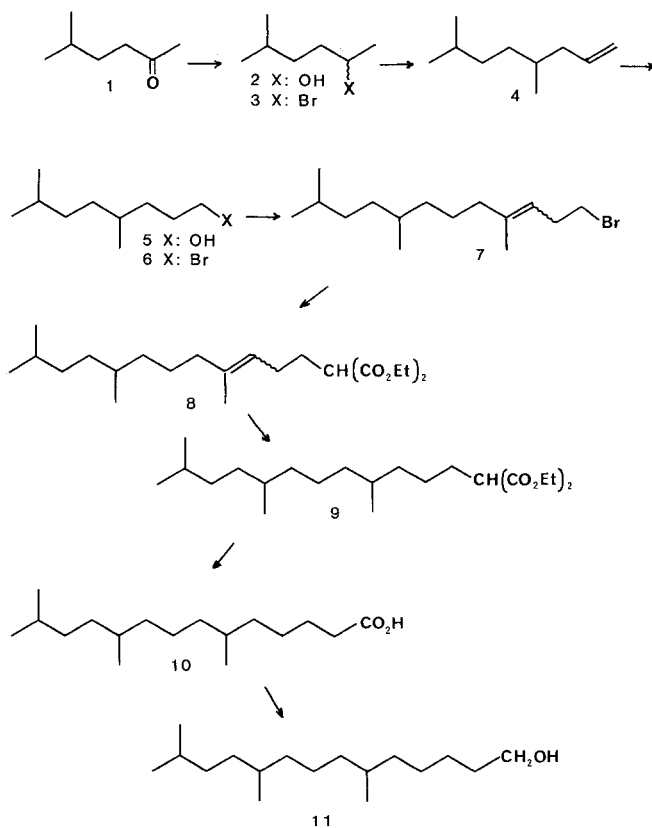


FIG. 1. Synthetic scheme for preparation of 6,10,13-trimethyl-1-tetradecanol.

METHODS AND MATERIALS

All potentially asymmetric compounds reported here are racemic. Solvents were of good technical grade, redistilled in glass. "Ether" was technical anhydrous ether redistilled from sodium, "dry" ether and THF were freshly distilled from LAH. Reactions requiring an inert atmosphere were carried out under nitrogen. Solutions of crude products were dried with $MgSO_4$. Temperatures are uncorrected. Structural confirmations were generally via GLC-MS since NMR and IR spectrometry are not very helpful with saturated aliphatic derivatives.

Chromatography and Instrumentation. Gas-liquid chromatographic (GLC) analysis of synthetic intermediates was carried out on a Packard-Becker model 421 fitted with a 30-m \times 0.53-mm DB-1 column (J&W Scientific, Folsom, California), using helium as carrier gas, and a Perkin-Elmer LCI-100 integrator. Temperature was programmed from 100 to 300°C at 10°/min. Comparisons with natural products were run on a Varian 3700 instrument with 14-m \times 0.25-mm DB-1 or DB-5 columns, helium carrier, and a Shimadzu C-R3A integrator. Integrations were not corrected for differences in detector response.

Mass spectrometry was carried out on a Finnigan MAT 4510 fitted with a 30-m \times 0.32-mm DB-1 column (J&W Scientific) and an INCOS data system.

The preparative HPLC system was based on a PULSAfeeder model 7120-SE diaphragm metering pump (Interpace Corp., Rochester, New York), rated 5.6 liters/hr at 210 kg/cm². All wetted parts were of stainless steel, and the discharge line from the pump to the injector valve had a T fitting leading to a stainless-steel pulse dampener (series 100, Kemlon Products and Development Co., Houston, Texas). The injector valve was a Whitey SS-43Y6 fitted with a 10-ml injector loop made from 2.75 m of $\frac{1}{8}$ in. OD \times 0.085 in. ID (3.175 \times 2.16 mm) stainless tubing.

Columns were fabricated from 3-ft (917-mm) sections of seamless type 304 stainless pipe, 2 in. \times schedule 40 (52.4 mm ID, 60.2 mm OD) with welded-on flanges. Heads were prepared from blind flanges by machining a recess for a stainless frit (Mott Metallurgical Corp., Farmington, Connecticut), a $\frac{1}{16}$ in. deep mixing chamber behind the frit, and a $\frac{1}{8}$ -in. NPT socket for a Swagelok® adapter. Packing was ca. 1500 g of Davison grade 922 silica gel, dry packed and tamped. Pressure drop with 1:1 hexane-ether at 125 ml/min was approximately 18 atm.

The UV detector (fixed wavelength = 254 nm) was salvaged from a DuPont model 830 instrument and fitted with a preparative cell. Further information is available from the first author.

2-Bromo-5-methylhexane (3). To 500 ml of 95% EtOH in a 1-liter three-neck flask fitted with a mechanical stirrer, reflux condenser, and addition funnel was added 23 g (0.6 mol) of $NaBH_4$; then 150 ml (1.17 mol) of 5-methyl-2-

hexanone (Aldrich Chemical Co.) was added over 45 min. The solution boiled spontaneously and the addition rate was adjusted to maintain gentle reflux. After 2 hr additional heating under reflux, the solution was cooled, poured into water, and extracted three times with ether. The combined extracts were washed with water (2×), 5% NaOH, water, and bicarbonate, and then dried. The solvent was removed on a rotary evaporator to give a colorless oil, which was used directly for the next step (bp 144–152°C/762 mm, Aldrich Catalog says 148–150°C).

The oil was dissolved in 500 ml ether in the same apparatus except that the condenser was replaced with a thermometer dipping into the solution. The mixture was cooled in ice to <10°C, then 100 g PBr₃ (0.37 mol, 1.11 eq) was added dropwise over 1.5 hr, keeping the solution temperature between 5 and 10°C. The solution was stirred for one additional hour in the ice bath, and then at room temperature overnight (ca. 22 hr total). The product was isolated by pouring the solution onto ice and extracting twice with ether after separation of the original ether layer. The combined extracts were washed with water and saturated sodium bicarbonate (twice), dried, and distilled to give 128.7 g of the bromide **3** as a colorless oil, bp 55–59°C/25 mm (61% from the ketone). Bergmann et al. (1966) report 58°C/18 mm.

4,7-Dimethyl-1-octene (4). A 2-liter three-neck flask, Friedrich condenser, and dropping funnel were dried at 130°C, cooled under N₂, and fitted with a paddle stirrer. Magnesium turnings (24.3 g, 1 mol) were added, followed by 179 g (1 mol) of 2-bromo-5-methylhexane in ether totaling 600 ml. The addition of bromide took 45 min, and the mixture was heated under reflux for an additional 45 min. Freshly distilled allyl bromide (86.5 ml, 1 mol) was added dropwise at a rate to maintain good refluxing over 1¼ hr. The resulting solution was heated under reflux for 1¼ hr and stirred overnight at room temperature. The two-phase mixture (a heavy gray oil had separated overnight) was hydrolyzed with saturated NH₄Cl solution (150 ml) to the separation of a dense granular precipitate and filtered. The filtrate was distilled to a vapor temperature of 125°C to remove solvent and excess allyl bromide (odor), diluted to 500 ml with pentane, and percolated through a 25 × 300-mm silica gel column with pentane to remove polar impurities. The eluate was distilled bp 148–156°C/763 mm to give a colorless oil (39.8 g, 28%, major component 89% by GLC). A further 5.3 g of slightly less pure material was taken off from 156–170°C and treated with KOH to remove a trace of HBr. Total yield 45.1 g. MS (EI) 140 (0.3% M⁺), 125 (0.4%), 112 (0.4%), 98 (5%), 84, 69, 57 (100%) MS(CI, CH₄), 141 (30%, M+H), 139 (15%), 125 (22%), 99 (45%), 97 (20%), 85 (100%), 83 (88%), 71 (98%).

4,7-Dimethyl-1-octanol (5). A 1-liter three-neck flask, addition funnel, and condenser were dried at 130°C, assembled hot, and cooled under N₂. Dry THF (200 ml) was transferred to the dry flask using a double-tipped needle, followed

by 4,7-dimethyl-1-octene (45 g, 286 mmol of contained olefin). To the magnetically stirred solution was added 1 M BH_3/THF (160 ml, 68% excess, Alfa) over ca. 1 hr at room temperature, and the mixture was stirred for a further 3 hr at room temperature. After cooling in ice to $<10^\circ\text{C}$, 10 ml of water was added slowly (gas evolution), followed by 75 ml of 3 M NaOH. Hydrogen peroxide (30%, 75 ml) was then added at such a rate as to keep the temperature $<40^\circ\text{C}$; then the mixture was heated to $50\text{--}55^\circ\text{C}$ (some reflux) for 1 hr and stirred overnight. Extractive work-up with ether, removal of the solvent on a rotary evaporator, and rotary evaporation with 2×100 ml of *n*-heptane (to remove some *n*-BuOH from the decomposition of the BH_3/THF during storage) gave 44.5 g of a colorless, fragrant (rose-fatty) oil containing 84% of the desired product and 5.3% of the 2-isomer by GLC, along with other minor impurities which were not identified.¹

The reaction of the crude alcohol in pyridine with freshly prepared (acid and SOCl_2) 3,5-dinitrobenzoyl chloride gave a crude ester (87% pure by GC), which was crystallized twice from pentane at -20°C to give 65 g of purified ester, mp $37.5\text{--}39.5^\circ\text{C}$ (98.6% pure by GC). NMR: 0.8–0.9 (9H) CH_3 complex multiplet, 1.0–2.0 complex multiplet (CH , CH_2) 4.42 (2H) triplet (CH_2O), 9.15 (3H) ArH, complex multiplet. Considerable product remained in the mother liquor by GLC, but it could not be induced to crystallize. A comparison sample of 3,7-dimethyl-1-octyl 3,5-dinitrobenzoate was prepared from 3,7-dimethyl-1-octanol (Givaudan) and crystallized from pentane, mp $37\text{--}39^\circ\text{C}$, mixture mp $<25^\circ\text{C}$ (liquefied on trituration). The NMR was indistinguishable from that of the 4,7 compound.

Saponification of 60.4 g of this ester with 2 eq NaOH/MeOH, and aqueous work-up gave 26.5 g purified 4,7-dimethyl-1-octanol as a colorless oil, which was 97.8% pure by GC. This represents a 56% yield from the olefin, allowing for the sample of ester not saponified. The compound has been previously reported (Crombie et al., 1957).

4,7-Dimethyloctyl bromide (6). Triphenylphosphine dibromide was prepared from 53 g (0.2 mol) Ph_3P and 10.3 ml Br_2 in CH_2Cl_2 (250 ml), adding enough additional Br_2 to give a faint yellow color, keeping the temperature between 10 and 15°C . The purified 4,7-dimethyl-1-octanol (5), in 100 ml CH_2Cl_2 , was then added over 25 min below 10°C , and the solution was allowed to warm up to room temperature with stirring overnight. Water (200 ml) was added, followed by solid NaHCO_3 to neutralize the HBr. Extraction of the separated aqueous phase with CH_2Cl_2 and evaporation of the organic layers gave a sticky mixture of Ph_3PO and product, which was partitioned between 90%

¹ A reviewer has pointed out that the quantities of NaOH and H_2O_4 were inadvertently less than stoichiometric. It is probable that the yield would improve by use of sufficient reagents to complete oxidation/hydrolysis.

aqueous DMSO and petroleum ether. The DMSO layer was extracted twice more with petroleum ether; then the extracts were washed twice with 90% DMSO to remove Ph_3PO and once with water, and dried. Removal of solvent gave 42.1 g of almost colorless oil, which was passed through a column (2 × 30 cm) of silica gel with petroleum ether to remove polar impurities, then distilled; bp 112–114°C/26 mm, 34.7 g (93.7% yield) 97.5% pure by GLC. MS (EI, 70 V) 220, 222 (M^+ , trace), 205, 207 (trace) 177, 179 (trace) 163, 165 (trace), 149, 151 (28%), 69 (50%), 57 (100%).

4,8,11-Trimethyl-3-dodecen-1-yl Bromide (Mixed Isomers) (7). To a 250-ml three-neck flask with a magnetic stirrer, addition funnel, and Friedrich condenser, dried at 140°C and cooled under N_2 , was added 4.8 g Mg and, over ca. 30 min, the bromide (6) (34.2 g) in dry ether (ca. 150 ml). After heating under reflux for 1 hr further, methyl cyclopropyl ketone (17 ml, 0.17 mol) was added over $\frac{1}{2}$ hr and the solution refluxed for a further $\frac{1}{2}$ hr. The alkoxide solution was then transferred (double-ended needle) to a dropping funnel fitted to a magnetically stirred 500-ml three-neck flask containing 100 ml 48% HBr and cooled in an ice bath. The alkoxide was added over about 45 min, keeping the pot temperature < 10°C. After a further 30 min, 400 ml H_2O was added. Extractive work-up with ether, followed by washing with water and NaHCO_3 , then passage through a 2.5 × 30 cm silica gel column with petroleum ether gave 38.9 g of a colorless oil that was distilled, bp 175–178°C/26 mm, to give 28.6 g of a colorless oil (GLC: 31.7% *Z*, 56.4% *E*, and 7.8% of the hydrocarbon dimer from the Grignard preparation). The mixed bromides contained represent a 55% yield.

6,10,13-Trimethyl-1-tetradecanoic Acid (10). Sodiomalonic ester was prepared in 200 ml of absolute ethanol (stored over 3A molecular sieves) from 3.4 g sodium (0.15 mol) and subsequent addition of 24 g (0.15 mol, 22.8 ml) freshly distilled diethyl malonate. After stirring for $\frac{1}{2}$ hr, the 28.6 g of crude bromide **7** above was added over 15 min and the funnel was rinsed with 30 ml absolute alcohol. After $1\frac{1}{4}$ hr reflux (GLC analysis of an aliquot taken after 55 min indicated the absence of starting material), the slurry (NaBr) was cooled, hydrolyzed with 500 ml of iced $\frac{1}{3}$ -saturated NaHCO_3 (to buffer the NaOH from hydrolysis of excess sodiomalonic ester), and extracted with three portions of ether, which were washed with water (twice), then brine, dried and concentrated to give 47.9 g of a pale yellow liquid with the odor of the excess malonic ester.

This crude ester (**8**) was dissolved in 95% EtOH (about 150 ml total volume) and hydrogenated over Pd/C at an initial pressure of 50 psi in a Parr apparatus overnight. Filtration and rotary evaporation gave 41 g of a colorless oil.

The saturated ester (**9**) was dissolved in ca. 200 ml 95% EtOH, and a solution of 30 g KOH (2 equivalents)/30 ml H_2O was added. After 45 min heating under reflux, the starting ester was absent by GLC. Most of the ethanol

was removed on a rotary evaporator, and the solution was diluted with water and extracted three times, with petroleum ether with addition of enough EtOH to break the emulsion. Acidification of the alkaline solution with dilute H_2SO_4 and extractive work-up with ether gave 33.7 g of an almost colorless oil, which did not crystallize from pentane at $-100^\circ C$ overnight. GLC of the methyl esters showed almost exclusively the substituted malonic acid with ca. 3% of the decarboxylated product.

The crude acid (33.2g) was heated in an oil bath under N_2 at $175^\circ C$ until effervescence ceased, then distilled (Kugelrohr), bp $135-155^\circ C$ (bath temperature)/1.3 mm, to give 18.5 g (75% from the bromide mixture) of 6,10,13-trimethyl-1-tetradecanoic acid (**10**) as a colorless liquid 97.3% pure by GLC of the methyl ester. We could find no mention of this compound in the literature, but the 5,9,13 isomer boils at $128-130^\circ C/0.05$ min (Weichet et al., 1957) or $150-160^\circ C/1-2$ mm (Lukes and Zobáčová, 1957). A few grams of undistilled residue on methylation (CH_2N_2) consisted essentially of one compound having MS characteristics indicative of bis(4,7-dimethyloctyl)acetic acid derived originally from dialkylated malonic ester, which presumably had a retention time too long to show up in the GLC runs of **8** or **9**. MS of methyl ester (CH_2N_2) 284 (0.4, M^+), 269 (0.3), 252, 253 (0.9,0.6), 208 (30), 143 (10), 115 (15), 111 (15), 97 (15), 97 (15), 87 (40), 74 (100).

6,10,13-Trimethyl-1-tetradecanol (**11**). Distilled 6,10,13-trimethyl-1-tetradecanoic acid (15.5 g, 57 mmol) was reduced in ether with 3 g of LAH (79 mmol, 85% excess). Filtration of the hydrolyzed (3 ml H_2O , 3 ml 15% NaOH, 9 ml H_2O) reaction mixture and evaporation of the filtered solution gave 16 g of an almost colorless liquid, which was distilled (Kugelrohr) to give 13.3 g of a colorless oil, bp $120-127^\circ C/1.8$ mm. This represents a 90% yield from the acid and a 4% overall yield from 5-methyl-2-hexanone. Purity was 96.2% by GLC. NMR: 0.85 (12H, broad doublet, CH_3), 1.0-2.0 (complex multiplet, CH , CH_2), 3.63 (2H, triplet, CH_2O).

In the initial preparation, which provided the material for the field tests, the crude alcohol (1.1 g) showed several spots by TLC on silica gel (Et_2O -hexane 1:1). The 3,5-dinitrobenzoate was prepared (acid chloride + alcohol + pyridine) and purified by preparative HPLC on silica gel with 15-20% ether in hexane to give 1.1 g of purified ester, which showed only one spot on TLC. The pale yellow oil could not be crystallized at $-20^\circ C$ but was saponified with $KOH-H_2O-EtOH$ at reflux for 15 min followed by extractive work-up with ether to give 419 mg of an oil (96.6% pure by GLC).

The products from both preparations were identical to each other and to the natural product by GLC coinjection on DB-1 and DB-5 columns, and the mass spectra were identical to those in Aldrich et al. (1986).

6,10,13-Trimethyl-1-tetradecanal. Alcohol (**11**, 1 μ l) was oxidized with a slurry of ca. 10 mg pyridinium chlorochromate in 1.5 ml CH_2Cl_2 . After 15 min, the solution was diluted with 1:1 Et_2O -hexane to 4 ml and percolated

through a 2.5-cm layer of silica gel in a disposable pipet with 2 ml more of the same solvent. GLC showed a 3:1 mixture of aldehyde and starting alcohol. The retention time (coeluted with natural material from 15-m DB-1 and DB-5 capillaries) and GLC-MS (see under airborne trapping in Results, below) were identical to those of the natural material.

Behavior. Field testing was conducted from July 4 through July 23, 1987, in a 0.94-hectare fallow field surrounded by mixed coniferous/deciduous forest at the Beltsville Agricultural Research Center. Goldenrod (*Solidago* spp.), bush-clover (*Lespedeza* spp.), and smartweed (*Polygonum* spp.), were especially abundant. Traps were made from 20 × 20-cm transparent cylindrical containers (Tri-State Molded Plastics, Dixon, Kentucky) (Aldrich et al., 1984) and suspended in contact with foliage from stakes (ca. 1 m above ground). Six pheromone-baited and six unbaited control traps were deployed in a randomized complete block design. Pheromone traps were rebaited daily with 0.5 mg of 6,10,13-trimethyl-1-tetradecanol (100 µg/µl CH₂Cl₂) on a rubber septum (5 × 9 mm, Thomas Scientific, Philadelphia, Pennsylvania).

Airborne Pheromone Trapping. Groups of 20 field-collected male or female *S. anchorago* were coaxed into a 785-ml glass column for trapping of volatiles. Air was drawn by vacuum for 24 hr through a charcoal prefilter, over the bugs, and through 30 mg of activated charcoal sandwiched inside a Swinney filter holder (13-mm stainless steel, Thomas Scientific). Trapped volatiles were washed from the filter in 200 µl CH₂Cl₂.

A similar procedure was used for determination of release rate and gland contents. Five laboratory-reared males whose sternal gland setae were visibly wet with secretion were used. The males were coaxed into a 10-ml, Luer-tip, disposable, glass syringe (Becton, Dickinson; HYPAK; Ace Scientific Supply Co., East Brunswick, New Jersey) with a cotton-stoppered water bottle, a meal-worm pupa for food, and charcoal prefilter (3 mm ID × 8 cm) in place of the plunger. Air was drawn by vacuum (30 ml/min) through ca. 30 mg of activated charcoal in a Swinney filter holder (as above) inserted onto the tip of the syringe. The males were aerated for 7.5 hr at 23°C during mid-photophase, and volatiles were washed from the filter in 345 µl of CH₂Cl₂. Release rate was determined by GLC external standard comparison to the synthetic material. Subsequent extraction of secretion directly from the sternal gland setae with heptane (Aldrich et al., 1986) and similar GLC analysis was used to measure residual alcohol.

RESULTS

Attraction Data and Observations. Since *Stiretrus* adults and larvae were reluctant to enter traps, individuals within 1 m of traps were collected and counted. A total of 116 bugs were captured at baited traps; three were caught

at unbaited traps. Sixteen of the 116 *S. anchorago* at baited traps were larvae (Figure 2), including second, third, fourth, and fifth instars. Thirty-two bugs were collected on or inside pheromone-baited traps; the remainder were on nearby foliage, often feeding and/or mating. Significantly ($P < 0.05$) more males were caught per day than females ($X = 0.435/\text{trap}/\text{day}$ for males and $X = 0.201$ for females). Analysis of three-day means by date (Table 1) revealed that, except for the fourth three-day period, more adults were caught toward the end of the test period. The low number of adults collected during the fourth three-day interval (July 14–16) was weather related, as it was cool and/or rained on these days. The trend of increasing adult captures with date was significant for males (Table 1) and was accompanied by a decline in the capture of larvae (Figure 2), presumably caused by larval/adult molts during this period.

Airborne Trapping. These experiments showed that 6,10,13-trimethyl-1-tetradecanol is emitted by males, presumably by evaporation from the sternal setae, whereas in airborne extracts of females this alcohol is absent. In addition, GLC analysis of male-derived extracts revealed that there is about 10 times more of a minor component eluting prior to the alcohol in airborne samples ($X \pm \text{SEM} = 5.03 \pm 1.56\%$ of total aldehyde + alcohol, $N = 2$) than in extracts prepared by directly extracting the sternal gland setae of single males with heptane or CH_2Cl_2 as previously described ($X \pm \text{SEM} = 0.53 \pm 0.12\%$, $N = 3$) (Aldrich et al., 1986). A second analysis of effluvia from five males showed the aldehyde to comprise 6.77% of the mixture (average of four determinations

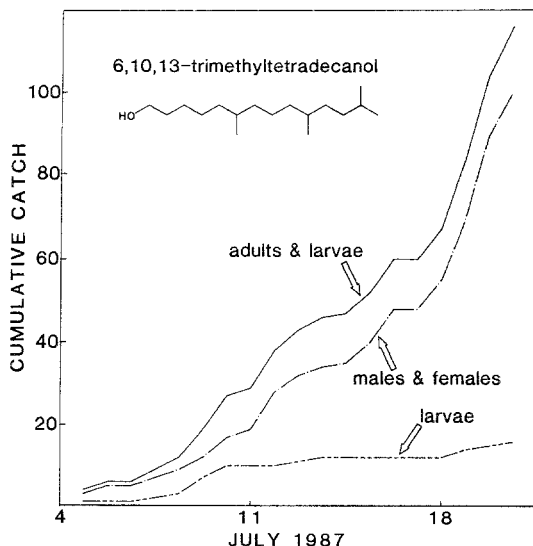


FIG. 2. Cumulative catch of *Stiretrus anchorago* adults and larvae at field traps baited with 0.5 mg 6,10,13-trimethyl-1-tetradecanol (traps rebaited daily).

TABLE 1. *Stiretrus anchorago* ADULTS CAUGHT AT FIELD TRAPS BAITED WITH 6,10,13-TRIMETHYL-1-TETRADECANOL

Interval ^a	X Adults/trap ^b	
	Male	Female
1	0.161 ab	0.042 a
2	0.176 ab	0.085 a
3	0.362 ab	0.309 a
4	0.085 b	0.176 a
5	0.607 a	0.224 a
6	1.515 c	0.399 a

^aIntervals are three-day summations beginning July 5, 1987.

^bCounts were square-root transformed for analysis by an *F*-least significant difference test ($P \leq 0.05$). Means within a column followed by a common letter are not significantly different. No adults were caught at control traps. Traps were baited with 0.5 mg trimethyl-1-tetradecanol and rebaited daily.

on the same sample) and the release rate to be 50 ng/male/hr, assuming continuous calling. Extraction of the sternal gland setae of the same five males gave 11.8 μg /male of an aldehyde/alcohol mixture containing 0.41% aldehyde. Methane CI-MS of the minor component showed an $[\text{M}-\text{H}]^+$ ion at m/z 255, suggesting that the compound is the corresponding aldehyde of the major alcoholic component. This supposition was verified by the identity of the EI-MS of the natural product [m/z (%): 55(33), 57(100), 69(27), 71(25), 82(16), 85(14), 95(30), 96(43), 109(16), 112(12), 123(4), 124(4), 126(5), 137(1), 165(1), and 168(1)] to that of the synthetic aldehyde and by the GC coelution of the synthetic aldehyde with the natural product ($RT = 5.27$ min, 135°C , 15 m DB-1).

Stiretrus adults frequently flew upwind toward traps while they were being rebaited, and flight activity was greatest on very hot days ($>35^\circ\text{C}$). The diet of *S. anchorago* appears to be more varied than previously suspected (Aldrich et al., 1986), as individuals were observed preying upon various caterpillars, a pentatomid larva, and a flatid, and both adults and larvae were seen probing flowers for nectar. Larvae of *S. anchorago* are decidedly beetle-like in appearance, an impression reinforced by the shiny black cuticle of most individuals. However, four fifth-stage larvae were collected whose abdomens were predominantly white. White *S. anchorago* morphs also occasionally occur in our laboratory colony.

DISCUSSION

The synthesis represents a straightforward elaboration of the molecule from the end remote from the functional group. 5-Methyl-2-hexanone is inexpensive and has the proper relationship of methyl groups. Reduction (NaBH_4) and con-

version to the bromide (PBr_3) proceeded in good yield. Preparation of the Grignard reagent and reaction with allyl bromide gave 4,7-dimethyl-1-octene, albeit in only 34% yield. In the initial preparation, this olefin was converted to the iodide by reaction with disiamylborane, then treatment with iodine and NaOH/MeOH by analogy with Brown (1975). The product after distillation was a colorless oil about 90% pure by GC, but the impurities caused subsequent problems. In the second run, hydroboration/oxidation of the olefin gave the alcohol (contaminated with the expected 6% 2-ol), whose 3,5-dinitrobenzoate could be purified by crystallization from pentane. Saponification and treatment with $\text{Ph}_3\text{PBr}_2/\text{CH}_2\text{Cl}_2$ gave 4,7-dimethyloctyl bromide of 97.5% purity (GLC) in excellent yield.

Initial attempts at 5-carbon homologation of this system involved the proposed reaction of 4,7-dimethyloctyl magnesium iodide with 5-chloro-2-pentanone to give a tertiary chloroalcohol, which could be employed directly in a malonic ester synthesis. Unfortunately, none of the desired product was obtained. Instead, a product more volatile than expected formed, whose GLC-MS properties were consistent with 2-methyl-2-(4,7-dimethyloctyl)tetrahydropyran. This can be rationalized by supposing that the Grignard reagent adds to the ketone to give a 3° alkoxide that displaces chloride to give the THP compound. This was not investigated further.

The Julia reaction proved satisfactory. Reaction of 4,7-dimethyloctyl Grignard with cyclopropylmethyl ketone and then with HBr , either by the two-step procedure (Julia et al., 1960) or the improved single step method (Biernacki and Gdula, 1979) gave a 55% yield of a 2-3:1 mixture of (*E*)- and (*Z*)-4,8,11-trimethyl-3-dodecen-1-yl bromide after distillation, whose major impurity was 8% of 2,5,12,15-tetramethylhexadecane from dimerization of the halide during the Grignard reaction (identification was by comparison of retention time with an authentic sample prepared from 4,7-dimethyloctyl iodide and sodium).

The rest of the synthesis was straightforward. Alkylation of sodiomalonic ester, hydrogenation, and saponification gave 4,8,11-trimethyldodecylmalonic acid. Decarboxylation and LiAlH_4 reduction gave the desired alcohol in an overall yield of 4%.

Attraction of *S. anchorago* to 6,10,13-trimethyl-1-tetradecanol in the field demonstrates that the male sternal gland secretion is an attractant pheromone and suggests that the same is true for other asopines with homologous glands (Aldrich and Lusby, 1986; Aldrich et al., 1986). Attraction of larvae to male-produced pheromones has been reported for other predaceous and phytophagous pentatomids (Aldrich et al., 1984; Harris and Todd, 1980), but the response of *S. anchorago* larvae to the pheromone released by males seems exceptionally pronounced. It is likely that *Stiretrus* males produce only one or some of the stereoisomers of the alcoholic pheromone (Silverstein, 1979), but unnatural isomers are apparently not inhibitory. Whether or not inclusion of the minor aldehydic component will increase trapping efficiency remains to be determined. Improvement of trap design is also probable. Because *S. anchorago* is a bene-

ficial insect, the difficulty in trapping them may not prevent the utilization of synthetic pheromone for augmentation and conservation of this predator in agroecosystems.

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FIELD RESPONSES OF CERTAIN FOREST COLEOPTERA TO CONIFER MONOTERPENES AND ETHANOL

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Abstract—Field experiments using baited sticky stovepipe traps and Lindgren multiple funnel traps were done near Chalk River, Ontario, Canada, to determine the effects of conifer monoterpenes (α -pinene, β -pinene, myrcene, limonene, camphene and carene) and ethanol on the number of beetles captured. Several species of conifer-feeding beetles were attracted to the monoterpenes or to monoterpenes and ethanol, including species in the families Cerambycidae (*Asemum striatum*, *Acmaeops proteus*, *Xylotrechus undulatus*, *Monochamus scutellatus*), Curculionidae (*Pissodes strobi*, *Hylobius pales*), and Scolytidae (*Dryocetes autographus*, *Ips grandicollis*). Species of Buprestidae generally did not respond to the monoterpenes or to ethanol. Species of Cleridae (*Thanasimus dubius*, *Enoclerus nigripes rufiventris*, *Enoclerus nigrifrons gerhardi*) which are predators of conifer bark beetles were attracted to the monoterpenes. Synergism between monoterpenes and ethanol was evident for *M. scutellatus*, *H. pales*, and *D. autographus*. While α -pinene was the most potent attractant for most beetle species, monoterpenes other than α -pinene synergized attraction to ethanol for *D. autographus*. Attraction of beetles to commercial turpentine and ethanol did not differ significantly from attraction to a pure monoterpene blend and ethanol.

Key Words—Kairomones, host attraction, monoterpenes, ethanol synergism, turpentine, trapping, Cerambycidae, Scolytidae, Curculionidae, Cleridae, Buprestidae, *Dryocetes autographus*, *Monochamus scutellatus*, *Hylobius pales*, Coleoptera.

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INTRODUCTION

With a few exceptions, such as certain species of *Dendroctonus* bark beetles, bark and wood-boring beetles associated with conifers attack weakened or dead trees. This fact generally is attributed to the monoterpenes and other terpenoid chemicals in the bark and foliage of conifers, which act as effective deterrents against feeding by beetles and other herbivores (Hanover, 1975; Cates and Alexander, 1982). For beetle species that are adapted to feeding on conifers, these same chemicals may act as kairomones, attracting beetles to their hosts.

While many species of conifer-feeding Scolytidae do not orient to host-produced chemicals, others are attracted to susceptible host material and to host resin or phloem (see Moeck et al., 1981; Wood, 1972, 1982). Pure conifer terpenes also have been found to attract scolytids, including *Hylastes ater* Paykull (Perttunen, 1957), *Ips grandicollis* (Eichhoff) (Werner, 1972a), *Tomicus (Blastophagus) minor* (Hartig) (Kangas et al., 1970), *Tomicus (Blastophagus) piniperda* (L.) (Perttunen et al., 1970; Byers et al., 1985; Schroeder and Eidmann, 1987), and other scolytids (Rudinsky, 1966; Chararas, 1979). Ethanol, produced by fermentation in moribund trees (Graham, 1968), may attract conifer-feeding species such as *Gnathotrichus sulcatus* (LeConte) (Cade et al., 1970; Moeck, 1971), *Trypodendron (Xyloterus) domesticus* (L.) (Kerck, 1972; Nijholt and Schönherr, 1976), *Trypodendron lineatum* (Olivier) (Graham, 1968; Moeck, 1970, 1971; Nijholt and Schönherr, 1976), and *Xyleborus saxeseni* Ratzeburg (Moeck, 1971). Ethanol may synergize attraction to host terpenes such as α -pinene, as seen with *T. lineatum* (Bauer and Vité, 1975; Nijholt and Schönherr, 1976), *Hylastes nigrinus* (Mannerheim), and *Pseudohylesinus nebulosus* (LeConte) (Nijholt and Schönherr, 1976), or to pine turpentine, as seen with *Dendroctonus terebrans* (Olivier) (Fatzinger, 1985). Monoterpenes, including α -pinene, myrcene and camphene, as well as ethanol can synergize attraction to pheromones in several species of Scolytidae (see Borden, 1982; Wood, 1982).

Monoterpenes and ethanol also may affect the orientation of beetles in families other than Scolytidae. In noncontrolled experiments, turpentine appeared to attract many species of Cerambycidae, Scolytidae, Curculionidae, and Cleridae (Gardiner, 1957) and species of Buprestidae, Cleridae, and Trogositidae (Wickman, 1969). Sawyers (*Monochamus* spp.) and pine reproduction weevils were attracted to turpentine in the field, and attraction was synergized by ethanol (Fatzinger, 1985). The weevil *Hylobius pales* (Herbst) was attracted to the host terpenes (+)- α -pinene and α -phellandrene in olfactometer tests (Thomas and Hertel, 1969) and to various monoterpenes released with ethanol in the field (Siegfried, 1987). Monoterpenes and ethanol acted synergistically to attract *Hylobius abietis* (L.) to traps (Tilles et al., 1986). Among the cerambycids, *Hylotrupes bajulus* (L.) was attracted to α -pinene in the laboratory (Becker,

1962), and *Monochamus alternatus* Hope was attracted to traps baited with monoterpenes alone and with monoterpenes and ethanol (Ikeda et al., 1980, 1981). Upwind flights in laboratory olfactometers indicated responses of the clerid *Thanasimus dubius* (Fabricius) to α - and β -pinene (Mizell et al., 1984). Ethanol might also be involved in the attraction of cerambycids and other beetles. Certain species of Cerambycidae, Cleridae, and Scolytidae were captured by traps baited with ethanol in an oak forest (Montgomery and Wargo, 1983).

Because of the many factors affecting beetle attraction, behavioral responses to semiochemicals recorded in the laboratory may vary from the responses to the same compounds in the field (e.g., Werner, 1972b). As is evident from the discussion above, few field experiments have been done to verify the responses to host volatiles for beetles other than scolytids and weevils. In the case of species thought to be attracted to turpentine, the monoterpenes responsible for the attraction have not been identified. Trapping experiments were therefore done to determine the effects of monoterpenes and ethanol on the attraction of forest Coleoptera in the field.

METHODS AND MATERIALS

Trapping was done on the grounds of the Petawawa National Forestry Institute near Chalk River, Ontario, Canada. The forests in the area are part of the Central Ottawa District of the Great Lakes-Saint Lawrence Forest Region (Rowe, 1972), and are locally dominated by natural stands of eastern white pine (*Pinus strobus* L.), red pine (*Pinus resinosa* Aiton), and jack pine (*Pinus banksiana* Lambert), and plantations of red pine and of larch (*Larix* spp.) and spruce (*Picea* spp.) hybrids. No major insect or disease problems were observed in the area.

Sticky stovepipe traps (pipe traps) (Chénier and Philogène, 1989) and eight-funnel Lindgren multiple funnel traps (funnel traps) (Lindgren, 1983) were used. Candidate monoterpenes were selected to obtain a terpene blend similar to that occurring in most eastern Canadian species of the Pinaceae, based on published identities of the terpenes of these trees (Guenther, 1949; Drew and Pylant, 1966; and many others). The monoterpenes used in the baits were (\pm)- α -pinene (97% pure), (-)- β -pinene (98%), (\pm)-camphene (95%), (\pm)-limonene (95%), and myrcene (89%) (Aldrich Chemical Co., Milwaukee, Wisconsin) and (+)-3-carene (90%) (ICN Biomedicals Inc., Plainview, New York).

Experiment 1: Effects of Monoterpenes and Ethanol. Trapping was done with baited pipe traps to compare the following four treatments: (1) unbaited control, (2) 95% ethanol, (3) monoterpenes, and (4) monoterpenes and ethanol.

Ethanol baits consisted of two 20-ml scintillation vials filled with 95% ethanol; vials were capped with inverted specimen vial caps with four 2-mm

holes in the sides of each cap. Two similarly capped vials were used as dispensers for the monoterpene blend, which consisted of 65% α -pinene, 20% β -pinene, 5% limonene, 5% myrcene, and 5% camphene. A 10 \times 75-mm lamp wick was placed inside each monoterpene vial to provide a greater and more constant rate of vaporization. For traps baited with both monoterpenes and ethanol, two monoterpene vials and two ethanol vials were used.

The four treatments were replicated in four blocks in a randomized block design. The four traps in a given block were at the corners of a square measuring 20 m per side. Traps were at least 30 m from the forest-field boundary. Two blocks were in the forest and two were in open fields, thereby sampling species restricted to any one of the habitat types as well as those species common to both areas (Chénier and Philogène, 1989). Site did not affect the nature of the responses to the baits (unpublished data).

Trapping was done from June 12 to August 5, 1986. Every two weeks (12- to 15-day intervals), large insects were collected from the traps. The positions of the four treatments in each block were randomly changed, moving the entire traps to their new positions. Weighed vials of freshly prepared baits were attached to the top of the traps, and the vials from the previous trapping period were reweighed to determine the quantity of chemicals released during the trapping period. After four weeks and at the end of the experiment, the Stikem Special (Seabright Enterprises, Emeryville, California) and all captured insects were removed from the traps; a fresh layer of Stikem was applied to the traps after the fourth week of trapping. Insects were cleaned and identified to species (conifer-feeding beetles and their associates) or family (other Coleoptera).

For each species, the number of individuals captured per treatment in each block was pooled over all trapping periods to minimize trap position effects. Since data in this and in the following experiments did not satisfy the requirements for parametric testing, nonparametric tests were used to evaluate results. Data were analyzed using Friedman tests corrected for ties (Zar, 1984, equation 13.38), verifying significance for $\alpha = 0.10$, $\alpha = 0.05$, and $\alpha = 0.01$. Significance of the differences between the rank sums of the four treatments was verified using Scheffé-type multiple contrasts with $\alpha = 0.10$, $\alpha = 0.05$, and $\alpha = 0.01$ [using standard error terms corrected for ties (Zar, 1984, equation 13.48)] and nonparametric analogs of Newman-Keuls multiple comparisons with $\alpha = 0.05$ [using standard error terms corrected for ties (Zar, 1984, equations 13.42 and 13.48)].

Experiment 2: Effects of α -Pinene, Minor Monoterpenes, and Ethanol. In order to distinguish between the attraction due to α -pinene and that due to other (minor) monoterpenes, an experiment was done with eight treatments: (1) unbaited control, (2) 95% ethanol, (3) minor monoterpenes, (4) ethanol and minor monoterpenes, (5) α -pinene, (6) α -pinene and minor monoterpenes, (7)

ethanol and α -pinene, and (8) ethanol, α -pinene, and minor monoterpenes. The minor monoterpene blend consisted of 45% β -pinene, 15% 3-carene, 15% limonene, 15% myrcene, and 10% camphene.

Ethanol baits consisted of two 11-ml vials each containing 10 ml of 95% ethanol; each vial cap had one 3-mm hole in the top. Two 4-ml vials with a 7-mm hole in each of the caps were used as dispensers for the α -pinene (4 ml/vial). Minor monoterpene baits consisted of 4 ml of the minor monoterpene blend in one 4-ml vial with a pierced cap (7-mm hole). Traps baited with the ternary treatment (treatment 8) were therefore baited with two ethanol vials, two α -pinene vials, and one minor monoterpenes vial.

The experiment was done in open fields. A randomized block design was used, with the eight treatments replicated in 10 blocks, using pipe traps in five of the blocks and funnel traps in the other five blocks. Trap type did not affect the nature of the responses to the baits, as indicated by data obtained in preliminary experiments and analyzed by the group concordance method of Schucany and Frawley (Zar, 1984). The eight traps in a given block were placed in two parallel lines with 20 m between the lines and 20 m between traps in a line. Traps were placed at least 40 m from the forest-field boundary.

The experiment ran from June 15 to August 16, 1984. Traps were serviced weekly (5- to 11-day intervals, depending on the weather). The positions of the eight treatments in each block were randomly changed, moving the entire traps to their new positions in the block. Bait vials were replaced weekly. On July 29, August 9, and August 16, the volumes of chemical baits remaining from the previous trapping period were measured to determine elution rates. All insects were collected weekly from the funnel traps. Large insects were collected weekly from the pipe traps, and a fresh layer of Stikem was applied to the pipes after the fourth week. All insects were collected from the pipe traps after the fourth week and at the end of the experiment. Conifer-feeding beetles and their associates were identified to species and counted.

The number of individuals captured per treatment was pooled within each block over all trapping periods. Data were analyzed as in Experiment 1.

Experiment 3: Effects of Commercial Turpentine and Pure Monoterpene Blend. A small-scale study was done to compare attraction to commercial turpentine with attraction to the monoterpene blend used in Experiment 1. Pipe traps were baited with: (1) 95% ethanol and the monoterpene blend, or (2) 95% ethanol and commercial turpentine (Record Chemical Company Inc., Montréal, Québec). Bait dispensers and trapping procedure were as in experiment 1. The two treatments were replicated in four blocks. Traps in a block were 20 m apart and were at least 60 m from traps in adjacent blocks. The experiment extended over three trapping periods, from July 7 to August 5, 1986. Since trap position effects were unlikely in this two-treatment test, data from each trapping period

were treated as replicates for each of the four blocks, giving a total of 12 replicates. Data were analyzed by Wilcoxon signed-ranks tests for paired-samples experiments (Zar, 1984, section 10.4).

RESULTS

Rates of vaporization of the chemical baits for all three experiments are given in Table 1. In experiment 2, given the composition of the minor monoterpene blend and the elution rates for the α -pinene and for the minor monoterpenes, terpenes were released from traps baited with both α -pinene and minor monoterpenes at an average rate of 0.59 g/day/trap and in a ratio of about 73% α -pinene, 12% β -pinene, 4% each of carene, limonene, and myrcene, and 3% camphene.

Experiments 1 and 2. Results are shown in Table 2 for experiment 1 and in Table 3 for experiment 2. Species of Buprestidae generally did not respond to the chemicals tested. Experiment 1 did not yield significant results for *Buprestis maculativentris* Say, but data from experiment 2 indicated treatment effects. However, the ranks of neither ethanol-baited traps (lowest rank) nor traps baited with α -pinene and ethanol (highest rank) were significantly different from control traps, and it appears unlikely that the treatment effects were of biological significance. For the melandryid *Serropalpus* sp., traps baited with the complete monoterpene blend (α -pinene and minor monoterpenes) and

TABLE 1. RATES OF VAPORIZATION^a (G/DAY/TRAP) OF BAITS, CHALK RIVER, ONTARIO, CANADA

Experiment	Type	Terpenes			Ethanol		
		Mean	SD	N	Mean	SD	N
1. June 12–August 5, 1986 ^b	blend	0.94	0.46	31	1.84	0.48	32
2. June 15–August 16, 1984 ^c	α -pinene	0.43	0.09	30	1.82	0.37	30
	minor terpenes	0.16	0.05	30			
3. July 7–August 5, 1986 ^b	blend	1.59	0.61	8	2.57	0.53	16
	turpentine	1.36	0.49	8			

^aMean, standard deviation (SD) and sample size (N).

^bDispensers (per trap): terpenes, two 20-ml vials with wicks; ethanol, two 20-ml vials.

^cDispensers (per trap): α -pinene, two 7-ml vials; minor terpenes, one 7-ml vial; ethanol, two 11-ml vials.

ethanol ranked highest in both experiments, with these compounds possibly acting synergistically as indicated by the Scheffé contrasts in experiment 1.

Four species of Cerambycidae showed definite responses to the treatments. In experiment 2, *Asemum striatum* (L.) was attracted by α -pinene, with minor monoterpenes exerting no apparent effect on attraction; while contrasts indicate a possible synergism between the α -pinene and ethanol ($P < 0.05$ for treatments with both compounds vs. other treatments), an examination of rank sums and multiple comparisons shows that the attraction was probably due simply to the presence of α -pinene ($P < 0.01$ for the contrast between treatments with α -pinene vs. treatments without). Both experiments indicated significant treatment effects for *Acmaeops proteus proteus* (Kirby), due largely to the presence of monoterpenes (experiment 1); rank sums and multiple comparisons indicate that the α -pinene may account for much of the attraction to the monoterpenes (experiment 2). *Xylotrechus undulatus* (Say) was attracted to monoterpenes in both experiments; in experiment 2, maximum attraction was obtained with traps baited with both α -pinene and the minor monoterpenes, and contrasts indicate a possible effect of the minor monoterpenes on attraction. Ethanol and α -pinene acted synergistically to attract *Monochamus scutellatus scutellatus* (Say), with the minor monoterpenes having little effect on this attraction (experiment 2); in the absence of ethanol, attraction to monoterpenes was significant only at $P < 0.10$.

Pissodes strobi (Peck) responded to the treatments in both experiments. In experiment 2, *H. pales* was attracted to traps baited with both α -pinene and ethanol, which acted synergistically.

Among the scolytids, almost all *Dryocetes autographus* (Ratzeburg) in experiment 1 were captured in traps baited with the combination of monoterpenes and ethanol. Experiment 2 demonstrated further that while α -pinene, minor monoterpenes, and ethanol alone did not attract this beetle, the combinations of α -pinene and ethanol and of minor monoterpenes and ethanol were very attractive ($P < 0.01$ for each of the relevant contrasts).

Treatment effects were detected for three species of clerid predators. *T. dubius* was captured in greatest numbers in traps baited with both monoterpenes and ethanol in experiment 1. Experiment 2 demonstrated further that the minor monoterpenes had no apparent effect on attraction, with most beetles being captured in traps baited with both α -pinene and ethanol. Both *Enoclerus nigripes rufiventris* (Spinola) and *Enoclerus nigrifrons gerhardi* Wolcott were attracted by α -pinene.

Experiment 3. None of the beetles showed significantly greater attraction to either the pure monoterpene blend and ethanol or to commercial turpentine and ethanol (Table 4).

TABLE 2. RESULTS OF FIELD TRAPPING EXPERIMENT TO DETERMINE EFFECTS OF MONOTERPENES AND ETHANOL ON CAPTURE OF BEETLES, CHALK RIVER, ONTARIO, CANADA, JUNE 12 TO AUGUST 5, 1986 (EXPERIMENT 1)

Family and Species ^a	Total number (#) and rank total (R) per treatment ^b				TE	Friedman chi-square ^c	Contrasts ^d				
	φ	E	T	TE			(T + TE) vs. φ	(T + TE) vs. (φ + E)	TE vs. (φ + E)	TE vs. (φ + E + T)	
Buprestidae											
<i>Dicerca tenebrosa</i>	# 6 R 11.5	3 7	4 8.5	9 13		5.00	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Buprestis maculativentris</i>	# 2 R 10.5	1 8.5	3 10	4 11		1.40	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Melanophila fulvoguttata</i>	# 0 R 7.5	0 7.5	5 12.5	6 12.5		6.25	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Chrysobothris dentipes</i>	# 2 R 9.5	1 8	8 13	2 9.5		5.40	n.s.	n.s.	n.s.	n.s.	n.s.
Other Buprestidae	# 5	12	20	15							
Cleridae											
<i>Thanasimus dubius</i>	# 2 R 5a	7 7a	37 12ab	98 16b		11.68***	0.05	0.01	0.01	0.01	0.10
<i>Thanasimus undulatus</i>	# 0 R 7a	1 8a	11 14a	9 11a		7.50*	n.s.	0.10	0.10	n.s.	n.s. ^e
<i>Thanasimus undulatus nubilus</i>	# 3 R 9.5	5 13	1 8	3 9.5		5.40	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Enoclerus nigrifrons gerhardi</i>	# 2 R 6a	4 7a	17 14a	19 13a		8.82***	0.10	0.05	0.05	n.s.	n.s. ^e
Other Cleridae	# 3	5	1	9							
Melandryidae											
<i>Serropalpus</i> sp.	# 1 R 7a	11 8ab	80 10.5ab	124 14.5b		8.04**	n.s.	n.s.	n.s.	0.05	0.10
Other Melandryidae	# 0	0	2	0							

<i>Enoclerus nigripes rufiventris</i>	#	6	13	10	15	18	19	26	12	18.51***	A(4vs4)**
	R	33.5a	43ab	35a	38.5ab	51ab	49ab	60b	50ab		
<i>Enoclerus nigrifrons gerhardi</i>	#	1	8	4	9	15	17	21	21	17.25**	A(4vs4)**
	R	33a	40ab	35ab	43ab	50.5ab	51ab	51ab	56.5b		
<i>Trichodes nutalli</i>	#	8	3	4	5	2	7	3	5	6.62	
	R	52	40.5	44	46	37	51.5	41	48		
Other Cleridae	#	8	9	4	10	5	8	4	7		
Melandyriidae											
<i>Dircaea quadrimaculata</i>	#	8	5	6	3	2	2	3	2	11.91	
	R	58	48	49	44	39.5	39	43.5	39		
<i>Serropalpus</i> sp.	#	0	5	4	12	6	16	21	18	15.19**	
	R	32a	41.5ab	39.5ab	41.5ab	45.5ab	55ab	49ab	56b		
Other Melandyriidae	#	2	3	2	2	2	3	1	1		
Cerambycidae											
<i>Asemum striatum</i>	#	3	2	2	0	16	12	22	14	32.79***	A(4vs4)***, AE(2vs6)**
	R	36.5a	34a	34a	32a	58b	46.5ab	66b	53ab		
<i>Rhagium inquisitor</i>	#	9	3	7	5	12	3	8	27	6.66	
	R	43	41.5	44	43	50	39.5	49	50		
<i>Acmaeops proteus</i>	#	1	7	4	7	4	13	24	6	19.84***	
	R	32.5a	44ab	40.5ab	46ab	41.5ab	52ab	63b	40.5ab		
<i>Xylotrechus undulatus</i>	#	0	0	7	8	15	25	14	23	26.78***	A(4vs4)**, AM(2vs6)**,
	R	27.5a	27.5a	41.5ab	44.5ab	54.5ab	60b	43ab	61.5b		AortM(6vs2)**
<i>Monochamus scutellatus</i>	#	4	7	9	14	20	12	18	21	25.93***	AE(2vs6)***, A(4vs4)*,
	R	32a	32a	40.5ab	45ab	46.5ab	41.5ab	60b	62.5b		AortM(6vs2)*
Other Cerambycidae	#	7	13	15	19	16	15	9	9		
Curculionidae											
<i>Pissodes strobi</i>	#	0	5	1	1	4	2	4	7	14.72**	
	R	37.5a	51.5ab	40.5ab	41ab	43ab	41.5ab	46.5ab	58.5b		
<i>Hylobius pales</i>	#	4	1	3	10	9	8	22	32	29.72***	AE(2vs6)***, A(4vs4)**
	R	36.5a	32.5a	34a	40.5a	45.5ab	41.5a	64b	65.5b		
Other Curculionidae	#	8	4	8	15	5	4	6	3		

TABLE 3. Continued

Family and Species ^a	Total number (#) and rank total (R) per treatment ^b										Friedman chi-square ^c	Contrasts ^d	
	φ	E	M	ME	A	AM	AE	AME	AE	AME			
Scolytidae													
<i>Dryocetes autographus</i>	# 11	18	4	76	16	6	83	79					
	R 30a	37.5a	27a	65b	38a	24a	69.5b	69b					51.16*** AE(2vs6)***, ME(2vs6)***, E(4vs4)***
<i>Orthotomicus caelatus</i>	# 7	17	4	4	13	3	10	5					9.73
	R 47.5	41.5	41	40	43	41.5	58.5	47					
<i>Ips grandicollis</i>	# 0	0	5	4	7	2	4	3					16.24**
	R 37a	37a	42.5ab	45ab	61b	43.5ab	49.5ab	44.5ab					
Other Scolytidae	# 3	9	7	16	9	9	9	4					

^aSpecies of bark- and wood-boring and predatory families with a total of 24 or more individuals captured.

^bTreatments: φ = blank control, E = ethanol, M = minor monoterpenes, ME = minor monoterpenes and ethanol, A = α-pinene, AM = α-pinene and minor monoterpenes, AE = α-pinene and ethanol, AME = α-pinene, minor monoterpenes and ethanol. Eight treatments replicated in 10 blocks using sticky stovepipe traps (five blocks) and Lindgren multiple funnel traps (five blocks). Rank totals in a row followed by the same letter are not significantly different ($P > 0.05$), nonparametric multiple comparison test.

^cCorrected for ties. * for $P < 0.10$, ** for $P < 0.05$, *** for $P < 0.01$.

^dNonparametric Scheffé-type contrasts. * for $P < 0.10$, ** for $P < 0.05$, *** for $P < 0.01$. Letters indicate treatment effects contrasted, numbers indicate number of treatments involved in contrast: A(4vs4) = [A + AM + AE + AME] vs. [φ + E + M + ME], E(4vs4) = [E + ME + AE + AME] vs. [φ + M + A + AM], AE(2vs6) vs. [AE + AME] vs. [φ + E + M + ME + A + AM], ME(2vs6) = [ME + AME] vs. [φ + E + M + A + AM + AE], AM(2vs6) = [AM + AME] vs. [φ + E + M + ME + A + AE], AorM(6vs2) = [M + ME + A + AM + AE + AME] vs. [φ + E].

TABLE 4. TOTAL NUMBER OF BEETLES CAPTURED IN STICKY STOVEPIPE TRAPS BAITED WITH TURPENTINE AND ETHANOL OR WITH MONOTERPENE BLEND AND ETHANOL, CHALK RIVER, ONTARIO, CANADA, JULY 7 TO AUGUST 5, 1986 (EXPERIMENT 3)

Family and Species ^a	Turpentine and ethanol		Monoterpene blend and ethanol		N ^b	P ^b
	Number	T ^b	Number	T ^b		
Buprestidae						
<i>Buprestis maculiventris</i>	12	15	8	6	6	P = 0.50
<i>Chrysobothris dentipes</i>	9	22	3	6	7	0.20 < P < 0.50
Cleridae						
<i>Thanasimus dubius</i>	41	10	34	0	4	P = 0.20
<i>Enoclerus nigrifrons gerhardi</i>	10	2	11	4	3	^c
<i>Trichodes nutalli</i>	9	13	2	2	5	P = 0.20
Melandryidae						
<i>Serropalpus sp.</i>	47	20.5	35	7.5	7	0.20 < P < 0.50
Cerambycidae						
<i>Acmaeops proteus</i>	10	8	6	2	4	P = 0.50
<i>Xylotrechus undulatus</i>	11	29	4	7	8	0.10 < P < 0.20
<i>Monochamus scutellatus</i>	10	10	4	0	4	P = 0.20
Curculionidae						
<i>Hylobius pales</i>	42	31	27	14	9	0.20 < P < 0.50
<i>Pissodes strobi</i>	4	5	7	10	5	P ≥ 0.50
Scolytidae						
<i>Hylastes porculus</i>	5	6.5	6	8.5	5	P ≥ 0.50
<i>Dendroctonus valens</i>	6	7.5	4	2.5	4	P ≥ 0.50
<i>Trypodendron lineatum</i>	1	0	11	1	1	^c
<i>Dryocetes autographus</i>	58	9	71	12	6	P ≥ 0.50
<i>Dryocetes affaber</i>	7	3	5	0	2	^c
<i>Ips pini</i>	21	13	11	2	5	P = 0.20

^aSpecies with 10 or more individuals captured.

^bWilcoxon signed-ranks test T, N (number of nonzero differences), and P (significance probability).

Two treatments replicated 12 times.

^cWilcoxon N too low to determine significance probability.

DISCUSSION

The general lack of response of Buprestidae to the various baits indicates that ethanol and the monoterpenes tested in these experiments probably do not play a major role in the orientation of these beetles. Buprestidae have also been shown not to respond to ethanol-baited traps in an oak forest (Montgomery and Wargo, 1983). Since most of the species of Buprestidae identified in this study were captured in greater numbers in traps with dark vertical silhouettes rather

than in clear flight interception traps (Chénier and Philogène, 1989), visual cues may be more important than chemical cues for the orientation of the species involved.

Results for *M. scutellatus* were similar to those obtained in Japan for *M. alternatus*. That species was attracted to a blend of 10 monoterpenes, with ethanol synergizing attraction (Ikeda et al., 1980). Among the monoterpenes, α -pinene proved the most attractive, with some activity being detected for β -pinene and β -phellandrene (Ikeda et al., 1981). In Florida, the sawyers *Monochamus carolinensis* (Olivier) and *Monochamus titillator* (Fabricius) were captured in greatest numbers in traps baited with both turpentine and ethanol, followed by traps baited with turpentine alone and with ethanol alone (Fatzinger, 1985).

The attraction of *H. pales* was greatest for traps baited with both α -pinene and ethanol. Siegfried (1987) reported attraction of *H. pales* to traps baited with α -pinene and ethanol, as well as attraction to β -phellandrene, limonene, and ethanol and to β -pinene and ethanol. Although Anderson and Fisher (1960) reported repellency of *P. strobi* by α -pinene and other terpenes, Wilkinson (1972) found that the weevil was attracted to white pine oleoresin. Experiment 1 clearly demonstrated attraction of *P. strobi* to monoterpenes.

Upwind flights in a laboratory olfactometer have shown that *T. dubius* responds to both α -pinene and β -pinene (Mizell et al., 1984). The greatest number of *T. dubius* in this study was captured in traps baited with both monoterpenes and ethanol.

Billings and Cameron (1984) found no attraction of *T. dubius*, *M. titillator*, or *I. grandicollis* to traps baited with loblolly pine turpentine, but the elution rates of the turpentine in their study ranged from 25 to 40 mg/day/trap, or about 25–40 times lower than the rates obtained in experiments 1 and 2. When turpentine was released from traps at rates of about 3.6 g/day, *T. dubius* was attracted to traps baited with frontalure and turpentine, but not to traps baited with turpentine alone; *M. titillator* was attracted to *Ips* pheromones and turpentine, but not to the pheromones or turpentine alone (Billings, 1985). The strong attraction to the pheromone–turpentine combinations possibly may have masked attraction of these two species to the turpentine. In the present study, both *T. dubius* and *M. scutellatus* were captured in greatest numbers in traps baited with both monoterpenes and ethanol. The effects of ethanol were not verified by Billings (1985). It is not known whether *M. scutellatus* is attracted to scolytid pheromones as is *M. titillator*. For beetles responding both to host odors and to scolytid pheromones, the strong responses to scolytid pheromones reported by Billings (1985) would permit efficient location of hosts; in the absence of pheromones, the weaker response to the host volatiles would still permit host finding. While Billings and Cameron (1984) and Billings (1985) used flight interception traps in their experiments, *T. dubius*, *M. scutellatus*, and other

beetles were captured in much greater numbers in vertical sticky stovepipe traps baited with monoterpenes and ethanol than in clear flight interception traps baited with the same chemicals (Chénier and Philogène, 1989), and it appears that visual cues may be of major importance for the orientation of these beetles to the tree volatiles.

Although results from experiment 3 indicate that attraction to the monoterpene blend and ethanol does not differ significantly from attraction to the chemically complex turpentine and ethanol, the small sample size should preclude definite conclusions. This is certainly the case with *T. dubius* and *M. scutellatus*, with Wilcoxon *T* values of zero for the monoterpene blend and ethanol (Table 4). Considering results from all three experiments, the use of commercial turpentine and ethanol as attractant baits could prove useful for sampling a wide variety of conifer forest Coleoptera.

While the responses of insects observed in these experiments are being heuristically referred to as "attraction", the true nature of the behavioral mechanisms involved is not known. The terpenes may be acting as arrestants, as was demonstrated with *Dendroctonus pseudotsugae* Hopkins exposed to phloem tissue of *Pseudotsuga menziesii* (Mirbel) Franco (Bennett and Borden, 1971). Most of the species that were found to respond to the test chemicals in these studies have been shown to land in greatest numbers on traps with distinct vertical silhouettes (Chénier and Philogène, 1989). Thus, while insects responding to the baits in these experiments may well be attracted from a distance, the chemicals may also be acting as arrestants for insects flying within sight of the traps.

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ALARM PHEROMONE PRODUCTION BY TWO HONEYBEE (*Apis mellifera*) TYPES

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Abstract—Of 12 alarm pheromones assayed in European and Africanized honeybees, nine were found in larger quantities in the Africanized population. Isopentyl and 2-heptanone levels were similar in both; 2-methylbutanol-1 was greater in European workers. These differences were not due to age or geographical location. Significant positive correlations between alarm pheromone levels and defensive behavior, especially numbers of stings, were observed.

Key Words—Honeybee, *Apis mellifera*, alarm pheromone, Hymenoptera, Apidae, isopentyl acetate, 2-heptanone, sting, mandibular gland.

INTRODUCTION

A vital part of honeybee colony defense is the communication of alarm through the use of pheromones (Maschwitz, 1964; Collins et al., 1980). Various investigators have identified alarm pheromones by chemical analysis and assay of biological activity. Boch et al. (1962) identified isopentyl acetate (IPA) (previously called isoamyl acetate) as a biologically active alarm pheromone associated with the sting, and Shearer and Boch (1965) reported on the alarm activity of 2-heptanone (2HPT) isolated from mandibular glands in the head. Blum and colleagues (Blum et al., 1978; Blum and Fales, 1988) identified over 80 compounds from the sting apparatus, of which 15 were shown to elicit alarm responses from caged workers (Collins and Blum, 1982, 1983). Pickett et al.

(1982) reported that (Z)-11-eicosen-1-ol was also an important component of the sting pheromone complex.

These initial identifications were made on samples of worker honeybees collected in North America (*Apis mellifera*, European subspecies). Two other subspecies, *A. m. scutellata* (formerly *adansonii*) and *A. m. capensis*, were assayed for IPA and 2HPT by Crewe and Hastings (1976). *A. m. scutellata* exhibits more intense colony defense than the European subspecies. The range of pheromone levels found, however, was within that seen in the European populations and did not prove to be an easily obtained predictor of defensive behavior.

Two other studies (Kerr et al., 1974; Boch and Rothenbuhler, 1974) investigated the relationship of IPA and 2HPT levels with several measures of defensiveness in the same colony of European bees. Levels of IPA did not show a significant correlation with the behavioral assay in either study, even though the more defensive line of Boch and Rothenbuhler had higher levels of IPA assayed. However, Kerr et al. (1974), using Africanized bees, reported that levels of 2HPT were positively correlated with three measures of defensive behavior: time to first sting, time to visible worker response, and number of stings.

The work reported here is from two studies measuring levels of IPA, 2HPT, and the more recently identified components of sting alarm pheromone (Blum et al., 1978; Collins and Blum, 1982, 1983) in two geographical types, European and Africanized bees.³ These geographical types display quite different levels of defensive behavior (Collins et al., 1982). One study involved sampling from a large number of representative colonies in Louisiana, U.S.A., and Monagas, Venezuela. The second was an experiment controlling for location and age in a smaller number of colonies. Correlations of the pheromone levels with aspects of defensive behavior as measured by a sequenced standardized test (Collins and Kubasek, 1982) were also calculated for the larger study.

METHODS AND MATERIALS

Survey. This experiment used 150 large colonies near Baton Rouge, Louisiana (30°30'N, 91°W), and 147 similar-sized colonies near Maturin, Monagas, Venezuela, (10°N, 63°W). The Louisiana colonies had been established

³The European honeybees in this study were from North America. Such bees have in their ancestry representatives of mixed subspecies. Africanized bees are descendants of *A. m. scutellata* bees imported from Africa and their hybrids with various subspecies previously imported into Brazil. Neither the European nor the Africanized bees can correctly be called race, subspecies, stock, or line representatives. We use the term "geographical type" to indicate that the bees we studied showed major characteristics typical of descriptions for temperately or tropically (*A. m. scutellata*) adapted bees.

from various U.S. commercial honeybee stocks. Some of the Venezuelan colonies had originally been headed by European queens but were chosen on the basis that they had not been requeened within at least the past year. This would have been sufficient time for several supersedures with the new queens mating with Africanized drones or for invasion of colonies by Africanized queens. The rest of the Venezuelan colonies had been established from caught swarms.

Ten bees from each colony were collected in alcohol and identified using an updated version of the Daly et al. (1982) morphometric identification procedure. Twenty-five characters were measured and the colonies classified as European, Africanized, or hybrid.

Samples of 30 worker bees of mixed ages per colony were collected from the entrance in a plastic bag and killed by freezing. The sting apparatus and head of each bee were removed from the body by forceps, immersed in 1 ml pesticide-grade methylene chloride with sodium sulfate as a drying agent, and sealed in a crimp-top vial. Three samples of 10 bees each were prepared per colony and stored in a freezer prior to analysis.

One-microliter aliquots of solvent from each vial were injected into a Perkin Elmer Sigma 3 gas chromatograph fitted with two glass columns (6 ft \times $\frac{1}{4}$ in. OD, 2 mm ID, packed with SP1000 on 80–100 mesh Chromasorb W with 10% loading) run as dual compensation. The heat cycle started at 70°C for 3 min and increased 5°C/min up to 120°C. Quantities of butyl acetate, IPA, 2-methylbutanol-1, hexyl acetate, 1-hexanol, 2-heptyl acetate, 2-heptanol, octyl acetate, 1-octanol, 2-nonyl acetate, 2-nonanol, and 2HPT were calculated by electronic integration using a Varian CDS III.

The data were analyzed with an analysis of variance for bee type using the mean value per colony. Colony means were also used for the correlations of pheromone and behavior measures. Data for each chemical were transformed by square root. Differences between means were determined by Tukey's test.

The colonies were also tested using a standardized procedure for measuring colony defense (Collins and Kubasek, 1982). These results were reported in Collins et al. (1982). For the defense test, a picture of the bees around the entrance was taken before testing. Timing of the test began when 0.8 ml of an artificial alarm pheromone (isopentyl acetate in paraffin oil, 1 : 100 by volume) was sprayed over the entrance. The other compounds from the sting had not been identified at the time of development of this test and were not used. At 30 sec, the second photograph was made, and the colony was given a physical jolt by shooting it with a glass marble (19 g, 2.3 cm in diameter) propelled by a slingshot. The third photograph was taken at 60 sec, and the suede targets were moved into place, one 2–6 cm in front of the entrance and a second 25 cm farther away. These two blue suede targets (5 \times 5 cm) were clipped to the arms of a battery-operated device that swung them vertically 20 cm about 120 times per minute during the 60- to 90-sec interval. At 90 sec, the targets were removed

and the final photograph was taken. New targets were used for each test sequence. At a later time, the number of bees in each photograph and the number of stings in the two targets were counted. Each colony was tested twice, on different days, and colony means were calculated.

Controlled Location and Age. Because samples from the extensive survey of the two populations were collected in separate geographic locations (Louisiana and Venezuela), the possibility existed that differences seen were due to environmental differences. Such factors as temperature, food sources, and previous disturbance might influence alarm pheromone levels or components produced. Also, both IPA and 2HPT levels vary with age, as reported by Boch and Shearer (1966, 1967), reaching a peak at ca. two weeks and then decreasing somewhat to a stable level. Whiffler et al. (1988) reported age variation for a number of the other alarm pheromones and associated changes in responsiveness to alarm pheromones.

To control for location and age in these pheromone assays, newly emerged bees from 10 Africanized and 10 European (various sources) colonies from the same apiary in Sarare, Lara, Venezuela (10°N, 69°W) were color-coded for eclosion date by being marked on the thorax with paint and released in the parent colony. Four weeks after emergence, marked bees were collected, held for 24 hr in a small cage in an incubator with sugar syrup and water available, and then killed by freezing. Stings were collected as above, except that only 0.5 ml of solvent was used and an internal standard, methyl octanoate, was added. Three additional sting alarm pheromones were measured, 1-acetoxy-2-octene, 1-acetoxy-2-nonene, and benzyl acetate. Data were analyzed by analysis of variance for bee type using the mean value per colony. Data were transformed by square root.

RESULTS AND DISCUSSION

Survey. All of the colonies in Louisiana were identified as European, but the population in Venezuela included 70 European colonies, 37 Africanized colonies, and six hybrid colonies. This allowed for additional comparisons of differences due to location (Europeans in Louisiana and Venezuela). Three Louisiana colonies and 29 Venezuela colonies were not precisely identified at the 0.90 threshold and were deleted from the data set.

The mean values of micrograms per bee produced for each of the 12 alarm pheromones are shown in Table 1. The sum of all the pheromones measured except 2HPT, which is not part of the sting pheromone complex, and 1-hexanol, for which there were too few observations, is also included. The four classes of bees were not significantly different for levels of IPA, the major sting alarm pheromone component. 2-Heptanone levels are probably not different in

TABLE 1. MEAN MICROGRAMS PER WORKER BEE (\pm SD) OF 12 ALARM PHEROMONES FOR TWO DISTINCT HONEYBEE TYPES AND THEIR HYBRID^{a,b}

Alarm pheromone	Bee type				<i>F</i> ^h
	European		Hybrid, Venezuela	Africanized, Venezuela	
	Louisiana	Venezuela			
Butyl acetate	0.01 \pm 0.03a ⁱ	0.14 \pm 0.22b	0.28 \pm 0.16c	0.56 \pm 0.35d	117.60**
Isopentyl acetate	1.92 \pm 0.85a	1.86 \pm 1.00a	2.10 \pm 1.13a	2.27 \pm 1.35a	1.42 NS
2-Methylbutanol-1	0.47 \pm 0.24a	0.43 \pm 0.23a	0.43 \pm 0.17ab	0.29 \pm 0.18b	8.63**
Hexyl acetate	0.04 \pm 0.03a	0.18 \pm 0.15b	0.33 \pm 0.18c	0.61 \pm 0.38d	135.12**
1-Hexanol	0.01 \pm 0.01a	0.02 \pm 0.03b	0.03 \pm 0.03b	0.10 \pm 0.06c	59.29**
2-Heptyl acetate	0.08 \pm 0.05a	0.14 \pm 0.08b	0.20 \pm 0.07bc	0.23 \pm 0.14c	30.97**
2-Heptanol	0.01 \pm 0.01a	0.02 \pm 0.02b	0.03 \pm 0.01c	0.06 \pm 0.04c	73.77**
Octyl acetate	0.02 \pm 0.03a	0.17 \pm 0.20b	0.28 \pm 0.24b	0.59 \pm 0.48c	91.84**
1-Octanol	0.01 \pm 0.02a	0.16 \pm 0.50b	0.27 \pm 0.12c	0.37 \pm 0.36c	61.88**
2-Nonyl acetate	0.03 \pm 0.05a	0.04 \pm 0.09a	0.06 \pm 0.09ab	0.12 \pm 0.11b	19.16**
2-Nonanol	0.14 \pm 0.18a	0.43 \pm 0.71b	0.64 \pm 0.27bc	1.93 \pm 4.44c	59.09**
2-Heptanone ^c	1.34 \pm 0.69a	1.01 \pm 0.53b	1.06 \pm 0.33ab	1.26 \pm 0.55ab	3.79*
Sum	1.46 \pm 0.33a	1.77 \pm 0.56ab	2.11 \pm 0.41bc	2.51 \pm 0.64c	4.85**
Number of colonies	64 ^d	70 ^e	6 ^f	37 ^g	

^a Sum is the total quantity of all sting alarm pheromones assayed, except 1-hexanol.

^b Based on colony means from 3 samples of 10 bees each. Transformed by square root for ANOVA and Tukey's.

^c This pheromone is produced in the mandibular glands, all others in the Koschewnikow gland associated with the sting.

^d Isopentyl acetate *n* = 144; 2-heptanone *n* = 146.

^e Hexanol *N* = 21.

^f Hexanol *N* = 3.

^g Hexanol *N* = 7.

^h ***P* < 0.01; **P* < 0.05; NS *P* > 0.05.

ⁱ Means within pheromone followed by the same letter are not significantly different.

the European and Africanized types, although the statistical relationships seen here are difficult to decipher. 2-Methylbutanol-1 is produced in greater quantity by European bees, but all the rest of the pheromones and their sum are at higher levels in the Africanized population. The intermediate values of the few hybrids indicate the likelihood of a simple additive genetic control for the production of most of these compounds.

Correlations of the levels of pheromones with the measures of colony defense for all colonies are presented in Table 2. The components of the sting alarm pheromone which occurred at higher levels in the Africanized group were consistently significantly correlated with a more defensive behavior. Levels of

TABLE 2. SIGNIFICANT CORRELATION COEFFICIENTS OF ALARM PHEROMONE LEVELS WITH DEFENSIVE BEHAVIOR IN STANDARD TEST FOR COMBINED POPULATION^a

Alarm Pheromone	Defensive behavior						Total stings
	Seconds to react to		Number of bees on entrance at:				
	Pheromone	Target	00 sec	30 sec	60 sec	90 sec	
Butyl acetate	-0.499	-0.466	0.148*	0.340	0.374	0.365	0.574
IPA	—	—	—	0.126*	0.142*	0.209	0.125*
Hexyl acetate	-0.521	-0.526	0.217	0.388	0.412	0.366	0.602
1-Hexanol	-0.292	-0.372	—	—	—	—	0.440
2-Heptyl acetate	-0.389	-0.379	0.234	0.321	0.337	0.373	0.463
Heptanol	-0.509	-0.417	0.140*	0.322	0.362	0.351	0.508
Octyl acetate	-0.450	-0.478	0.171*	0.345	0.369	0.313	0.556
Octanol	-0.417	-0.432	—	0.291	0.308	0.283	0.505
2-Nonyl acetate	-0.247	-0.180*	—	0.203	0.223	0.189	0.268
2-Nonanol	-0.452	-0.377	—	0.288	0.317	0.285	0.486
2-Heptanone	—	0.140	—	—	—	—	—
Sum	-0.428	-0.430	0.152*	0.334	0.368	0.378	0.554

^aSum is the total quantity of all sting alarm pheromones assayed except 1-hexanol. $P < 0.01$, except * = $P < 0.05$.

2HPT, the mandibular gland pheromone, do not correlate well with behavioral measures. IPA, which was not significantly different between bee types, shows only a low correlation with the number of bees responding to the test stimuli and with the number of stings. The corresponding alcohol, 2-methylbutanol-1, had no significant correlation with the behavior. Levels of 1-hexanol were not correlated with the number of bees responding but were correlated to the speed of the responses and to number of stings. The low correlations to number of bees on the entrance at 00 sec are expected since stimuli for defensive behavior have not been presented at this time.

Controlled Location and Age. From an examination of the data in Table 1, there are differences between the European groups from Louisiana and Venezuela. Nine of the compounds are at higher levels in the Venezuelan group. This difference can be easily explained by the presence of Africanized drifters in the Venezuela European colonies. Morphometric identifications support this, as only 12% of the Louisiana European colonies had one or two bees classed as Africanized, while 42% of the Venezuela European colonies had one to three Africanized individuals.

The levels of alarm pheromones found in worker bees of the two types when geographic location was identical and the age of the collected bees was controlled are shown in Table 3. The results are similar to those found for the initial survey. The major difference is that in the controlled group, IPA occurs in greater quantity in the Europeans. In comparing the actual quantities of IPA found per bee, the bees in the second study have less than half of what was found previously. IPA increases to a peak in bees that are of guarding and beginning-foraging age (ca. 2 weeks) and decreases thereafter (Boch and Shearer, 1966). In the age-controlled collection, the younger bees of guarding age with higher IPA levels were excluded and the 4-week-old bees collected would expectably show decreasing IPA levels.

The two values for 2-heptyl acetate and 2-nonyl acetate are not statistically different in the second study. However, the direction of the difference that is there (Africanized greater than European) is the same as that previously found. Of the three new pheromone components analyzed, 1-acetoxy-2-octene, 1-acetoxy-2-nonene, and benzyl acetate, only the last shows a significant difference with Africanized levels greater than European.

TABLE 3. MEAN MICROGRAMS (\pm SD) PER WORKER BEE OF 15 ALARM PHEROMONES FROM 4-WEEK-OLD BEES OF BOTH EUROPEAN AND AFRICANIZED TYPE REARED IN SAME APIARY^a

Alarm pheromone component	Bee type		
	European	Africanized	F ^c
Butyl acetate	0.06 \pm 0.05	0.66 \pm 0.05	73.71**
Isopentyl acetate	1.30 \pm 0.09	0.67 \pm 0.09	24.90**
2-Methylbutanol-1	0.07 \pm 0.02	0.01 \pm 0.02	5.05*
Hexyl acetate	0.10 \pm 0.05	0.74 \pm 0.05	95.15**
1-Hexanol	0.04 \pm 0.02	0.17 \pm 0.01	55.42**
2-Heptyl acetate	0.09 \pm 0.02	0.14 \pm 0.02	3.61 NS
2-Heptanol	0.10 \pm 0.01	0.16 \pm 0.01	12.75**
Octyl acetate	0.16 \pm 0.05	0.74 \pm 0.06	57.77**
1-Octanol	0.11 \pm 0.04	0.49 \pm 0.04	42.66**
2-Nonyl acetate	0.11 \pm 0.02	0.17 \pm 0.02	3.51 NS
2-Nonanol	0.33 \pm 0.12	1.40 \pm 0.13	38.14**
1-Acetoxy-2-octene	0.31 \pm 0.03	0.34 \pm 0.03	0.85 NS
1-Acetoxy-2-nonene	0.001 \pm 0.001	0.002 \pm 0.002	0.10 NS
Enzyl acetate	0.51 \pm 0.09	1.16 \pm 0.10	23.88***
2-Heptanone ^b	15.46 \pm 1.52	14.68 \pm 1.59	0.12 NS

^aTen colonies of each type were sampled.

^bSee Table 1, footnote ^c.

^c* $P < 0.05$; ** $P < 0.01$; NS, not significant.

Therefore, it appears that with the exception of IPA (the major sting alarm pheromone component) and its alcohol, 2-methylbutanol-1, and, possibly, 2HPT (a mandibular gland alarm pheromone), Africanized bees produce greater quantities of nine alarm pheromones. Furthermore, within the species *Apis mellifera*, these higher concentrations of alarm pheromones are associated with more intense levels of colony defense. The correlation of 0.554 (Table 2) between the total amount of alarm pheromone and the total number of stings indicates that 0.31 (r^2) of the variance of numbers of stings is determined in linear fashion with the variance in quantities of alarm pheromone (Snedecor and Cochran, 1967). A higher concentration of pheromone released from the first bee alerted could alert more recruits. Because alarm pheromones mediate the first step in honeybee colony defense (Collins et al., 1980), the influence of a higher concentration of pheromone on the intensity of the defensive response is easily seen and has been documented by Collins et al. (1987b). As the recruited bees also release pheromone through extrusion of the sting and loss of the sting in an intruder, a higher concentration of alarm pheromone would accumulate around Africanized colonies than around equivalent European colonies. This higher level of pheromone would also require a longer time to disperse. Both of these phenomena would account for at least part of the difference in defensive behavior between the two honeybee types. At this time, it does not seem necessary to invoke synergistic effects of differing proportions of the components in the two bee types to account for behavioral differences.

The correlations found between the pheromone produced and the behavior induced suggest that the genes controlling the variation of both of these characters are coadapted, that is, jointly selected for their effects on fitness (Falconer, 1981). It has been further hypothesized that coadapted genes form supergenes by being closely associated on a chromosome (linked) (Dobzhansky, 1970). Such supergene complexes would tend to stay together and could account for the persistence of the intensive African defense phenotype in the Africanized populations.

We have reason to believe that the number of genes involved could be small for each character. Both the behavior (Collins et al., 1984) and the pheromones (Collins et al., 1987a) show genetic correlations. A few genes could determine structure or biochemistry of the central nervous system, resulting in variable behavior. Likewise, very few genes might be needed for the synthesis of the array of very similar compounds that are the alarm pheromones studied.

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BIOCHEMICAL AND BEHAVIORAL EVIDENCE FOR
HYBRIDIZATION BETWEEN FIRE ANTS, *Solenopsis*
invicta AND *Solenopsis richteri* (HYMENOPTERA:
FORMICIDAE)

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Abstract—Behavioral and biochemical evidence is presented for hybridization between the fire ants, *Solenopsis richteri* and *S. invicta*. The response of the two species to extracts of their trail pheromones presented as a point source is clearly species-specific; however, hybrid workers responded to parental Dufour's gland extracts and parental workers responded to Dufour's gland extracts of the hybrid. The behavioral evidence for hybridization was confirmed by gas chromatograph comparison of the Dufour's gland extracts of the three fire ant forms, which showed a pattern for the hybrid that was intermediate to the two parental species.

Key Words—Fire ants, *Solenopsis invicta*, *Solenopsis richteri*, Hymenoptera, Formicidae, trail pheromone, hybridization.

INTRODUCTION

The discovery of hybridization between two imported fire ant species, *Solenopsis invicta* and *S. richteri*, in the United States has raised questions regarding the validity of their species status and the mechanism by which the extensive hybrid population is being maintained (Vander Meer et al., 1985; Ross et al., 1987; Vander Meer and Lofgren, 1988). Based on the earliest known records of imported fire ants in the United States (see Lofgren, 1986), it was deduced that a black form entered the Mobile, Alabama, area around 1918. A red form was also discovered in the Mobile area and was thought to have been introduced

in the 1930s. Both forms were identified as *S. saevissima* var. *richteri* until Buren (1972) elevated the black form to species status (*S. richteri*) and described the red form as a new species (*S. invicta*). These assignments were based on their lack of hybridization and the consistency of phenetic characters in the United States populations. However, hybridization between the two species has been recently discovered on the basis of biochemical characters (Vander Meer et al., 1985). Morphologically the hybrid is difficult to distinguish from *S. richteri* (Vander Meer et al., 1985; Ross et al., 1987). Biochemical analysis of museum specimens collected in the United States (Vander Meer and Lofgren, in preparation) indicates that hybridization has occurred wherever the range of the two forms has overlapped. Although this indicates that the criteria for differentiating them as species may no longer be valid, we will continue to use the species designations of Buren (1972).

The rapid spread of imported fire ants after their introduction was due primarily to their transportation by man in nursery stock and sod. The speed and mode of this spread resulted in an early disjunct distribution in Alabama, Louisiana, and Mississippi (see Bruce et al., 1949; Culpepper, 1953), which could not be explained by dispersion after natural mating flights. Currently the gaps have been filled, except at the western and northern frontiers of their range. At the present time, imported fire ants occupy 100,000,000 hectares in 11 states of the south and southeast and in Puerto Rico. Unfortunately, previous surveys of fire ant infestations did not distinguish between the red and black forms, so we know very little of the population dynamics of the two forms that have taken place since the 1940s.

Species-specific pheromones in social insects often play an important role in maintaining species isolation, both sexually and in foraging situations (Hölldobler and Carlin, 1987). In addition, the competitiveness of a colony or ant population is mediated to a large extent by its ability to discriminate the chemical cues associated with territory, recruitment, and competitor/enemy recognition (reviewed in Hölldobler and Carlin, 1987). In this paper we compare the behavioral species specificity of the recruitment pheromones produced by *S. invicta*, *S. richteri* and their hybrid, as well as the biochemical composition of these pheromones.

METHODS AND MATERIALS

Source of Ant Colonies. All individuals used as sources of Dufour's glands and for bioassays were taken from mature (producing sexuals) queenright, monogynous colonies. *Solenopsis invicta* colonies were collected from the field or reared from newly mated queens collected in Alachua County, Florida (see Banks et al., 1981, for methodology). Queenright *Solenopsis richteri* colonies

were collected along the Natchez Trace in Lee County, Mississippi. Hybrid colonies were collected from Lowndes County, Mississippi. The identification of the *S. richteri* and hybrid colonies was verified by gas chromatographic analyses of venom alkaloids and cuticular hydrocarbons (Vander Meer et al., 1985).

Source of Glandular Trail Pheromone Extracts. Dufour's glands from randomly chosen worker ants from each of the *Solenopsis* forms were extirpated in water, then transferred to, and macerated in a vial containing hexane (Burdick and Jackson, HPLC grade, Muskegan, Michigan). Pooled Dufour's gland extracts were used for both gas chromatographic (GC) analysis and bioassays. For bioassays, the concentration of the Dufour's gland extract was adjusted to give the desired number of worker equivalents (WE) per microliter by dilution with hexane or evaporation under a stream of nitrogen.

Gas Chromatograph Analysis. Hexane extracts or soaks were analyzed by GC on a Varian 3700 gas chromatograph (Sunnyvale, California), equipped with a flame ionization detector. Analysis of hexane soaks (1–24 hr) for venom alkaloids and cuticular hydrocarbons was performed with a 30-m DB-1 fused silica column (J&W Scientific, Inc., Folsom, California) and oven program of 150°C to 285°C at 5°/min (Vander Meer et al., 1985; Ross et al., 1987). Separation of the volatile components of the Dufour's gland extracts was achieved with a 15-m DB-1 fused silica column (J&W Scientific), operated isothermal at 88°C. Peak areas were integrated and the separation visualized on a Varian Vista 401 data processor.

Recruitment Pheromone Point Source Bioassay. One to three rearing cells from queenright laboratory-reared colonies were placed on top of each other in the center of a clean colony tray (7 × 44 × 56 cm). Ten positions were marked in a circle, 15 cm from the edge of the cells and equidistant from each other around the cells. Workers in the foraging arena of the donor colony tray were also transferred to the clean bioassay tray. The ants were given a minimum of 1 hr to habituate to their new surroundings. Squares of blotter paper (2 × 2 cm) on which a 1.5-cm-diameter circle had been drawn in the center were placed on larger squares of aluminum foil. The circular area was treated with a Dufour's gland extract (5 µl of hexane) containing one worker equivalent of one of the following: Dufour's gland extracts from *S. invicta*, *S. richteri*, or the hybrid (as defined by GC analysis; see Vander Meer et al., 1985; Ross et al., 1987), or a hexane control. The samples were randomly placed on the numbered locations around the colony cells. The number of ants within the 1.5-cm circle on the blotter paper squares were counted at 5-min intervals for 30 min. The sum of the six counts was regarded as the result for each position. Each bioassay was replicated using 10 different *S. invicta*, *S. richteri*, and hybrid colonies. Colony-to-colony variation was leveled out by setting the response of test colony workers to their own Dufour's gland extract at 100 and their response to the hexane control at 0 (see also Vander Meer et al., 1988).

RESULTS

Point-Source Bioassay. The response of foraging worker ants in the point-source bioassay to Dufour's gland extracts from *S. invicta*, *S. richteri*, and the hybrid are shown in Table 1. *S. invicta* responded poorly to the Dufour's gland extract of *S. richteri* and, similarly, *S. richteri* responded minimally to the Dufour's gland extract of *S. invicta*, demonstrating distinct behavioral differences for the two fire ant species in this bioassay. The response of workers from both parental species to hybrid Dufour's gland extracts was indistinguishable from the response of hybrid workers to their own Dufour's gland extract. Likewise, hybrid workers did not discriminate between the Dufour's gland extracts of the parental species or extracts of their own Dufour's glands.

Chemical Analysis of Dufour's Gland Extracts. The results of gas chromatographic analysis of Dufour's gland extracts from *S. invicta*, *S. richteri*, and hybrid are shown in Figure 1. The chromatographic trace of *S. invicta* has the characteristic profile previously reported (Vander Meer et al., 1981). (*Z,E*)-alpha-farnesene (A) is the major peak followed by an as yet unidentified mono-unsaturated tricyclic homosesquiterpene (B) (Vander Meer et al., 1988) and two known acyclic homosesquiterpenes (C and D) (Alvarez et al., 1987). The *S. richteri* profile is characterized by a single major component that has been shown to be identical to component B in *S. invicta*. However, none of the minor components in the *S. richteri* GC trace correspond to the identified biologically active components observed for *S. invicta*. The Dufour's gland GC trace of the hybrid shows a combination of all four known biologically active recruitment pheromones (A, B, C, and D) in proportions indicative of a blend of the two parental species. Components A, C, and D, absent from the Dufour's glands of *S. richteri*, are present in the hybrid, and component B, which is found in only trace amounts from Dufour's glands of *S. invicta*, is conspicuously present in the GC trace of the Dufour's gland of the hybrid.

TABLE 1. PERCENT RESPONSE OF FIRE ANT WORKERS IN POINT-SOURCE BIOASSAY OF DUFOUR'S GLAND EXTRACTS^a

Test species	Dufour's gland extract ^b		
	<i>S. invicta</i>	<i>S. richteri</i>	Hybrid
<i>S. invicta</i>	100.0	32.1 ± 7.7	123.3 ± 16.2
<i>S. richteri</i>	16.1 ± 1.8	100.0	103.2 ± 7.3
Hybrid	109.1 ± 18.9	147.3 ± 17.2	100.0

^aResults are expressed as the mean and standard error of 10 replicates.

^bTest samples were applied at a concentration of 1 WE/5 µl solution.

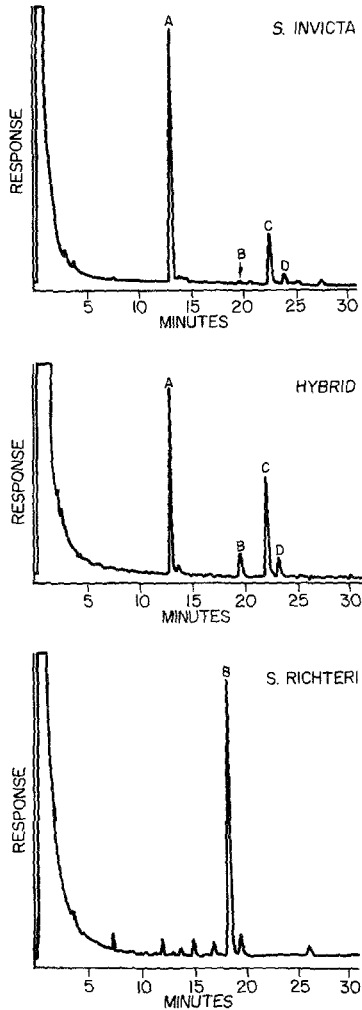


FIG. 1. Gas chromatograms of the volatile region from Dufour's gland extracts that have recruitment pheromone activity. See Methods and Materials section for details of the GC conditions.

DISCUSSION

Species-specific semiochemicals play an important role in many facets of social insect behavior, for example, reproductive isolation in bumblebees (van Honk et al., 1978) and recruitment of worker ants to food resources (Morgan, 1984; Attygale and Morgan, 1985). In some ant species, colony-specific cues (*Lasius*

neoniger) (Traniello, 1980) and even individual-specific orientation trails (*Pachycondyla tesserinoda*) (Jessen and Maschwitz, 1986) have been discovered. Although nonsemiochemical factors also affect species separation, the partitioning of resources in complex ant communities is largely dependent on chemical communication employed in territorial and recruitment behavior (Hölldobler and Carlin, 1987).

Fire ants have a complex food foraging and recruitment mechanism. Following food discovery, the foragers use light as a visual orientation cue to guide them to the entrance to their foraging tunnel. On the return trip, the forager leaves a trail by touching its stinger to the substrate and simultaneously releasing pheromone that originates in the Dufour's gland (Wilson 1959, 1962; Vander Meer, 1986). At the nest, additional pheromone is released, which induces other workers to follow the trail (Vander Meer, 1986; Vander Meer et al., unpublished). Depending on the size and quality of the food source, the returning workers reinforce the trail, thus providing a feedback mechanism regulating the number of workers recruited (Wilson, 1962).

The fire ant recruitment process has been reduced to three measurable and sequential behaviors: (A) attraction, (B) orientation induction, and (C) orientation (Vander Meer, 1986). Orientation has been demonstrated to be nonspecific for the two species (Barlin et al., 1976; Jouvenaz et al., 1978). Orientation induction shows an asymmetric specificity (Vander Meer, 1986; Vander Meer and Lofgren, unpublished). However, worker response to a point source of Dufour's gland extract is species-specific (Vander Meer, 1986). The latter bioassay measured attraction and aggregation to Dufour's gland extracts (Vander Meer et al., 1988). Quantitative differences in active components present in the Dufour's glands of the two species (Figure 1), coupled with differential worker sensitivity to those components, could account for the observed non-specificity of the orientation and attraction bioassays (Barlin et al., 1976; Jouvenaz et al., 1978), as well as the species specificity of the point-source bioassay (Vander Meer, 1986).

Our results using the species-specific point-source bioassay mentioned above (Table 1) clearly provides the first behavioral evidence for hybridization between *S. invicta* and *S. richteri*. Although the chemistry associated with the three recruitment behavioral subcategories is not fully known, it is clear from a visual comparison of the gas chromatograph traces (Figure 1) of the hybrid and its parents that the hybrid combines the chemical features of its parents. This is analogous to the gas chromatograph profiles of the hybrid and parent venom alkaloids and hydrocarbons that were first used to describe the hybrid (Vander Meer et al., 1985; Ross et al., 1987).

Recent surveys in Mississippi, Alabama, and Georgia indicate that the range of the hybrid population is extensive throughout the northern portions of

these states (Vander Meer and Lofgren, 1988; Diffie et al., 1988). The only place that the hybrid is sandwiched between its two parental types is in northeastern Mississippi. At the present time competition for territory is taking place between (1) hybrid and *S. invicta*; (2) hybrid and *S. richteri*; and (3) hybrid and hybrid, but not between the two parental types.

Chemical analyses of fire ant samples from the Meridian, Mississippi, area over a 24-year period of time indicates that the hybrid and *S. invicta* have reached a point of equilibrium, with neither form capable of displacing the other (Vander Meer and Lofgren, unpublished). In contrast, chemical analyses at the hybrid-*S. richteri* interface indicate that the hybrid is displacing *S. richteri*. It is probably only a matter of time before *S. richteri* is eliminated from the United States.

Analysis of alcohol-preserved fire ant specimens for their venom alkaloid patterns demonstrated that hybridization between *S. invicta* and *S. richteri* was taking place wherever and whenever the two species have met (Vander Meer and Lofgren, in preparation). Consequently, all three forms have been vying for territory over the last 50 years. It could be argued that the lack of recruitment specificity of the hybrid would give it a homospecific versus a heterospecific advantage in resource competition with either of the parent species. However, the current distribution suggests that other factors, such as environmental and ecological adaptability, have probably played an important role in the observed population dynamics of *S. invicta*, *S. richteri*, and their hybrid. In South America, *S. invicta* occupies the northern tropical and subtropical areas, whereas *S. richteri* occupies the southern, more temperate climatic zone (Buren, 1972). The same climatic distribution is currently seen for the two parental types in the United States. The hybrid occupies a climatic niche in between their parents. To arrive at the present hybrid distribution requires: (1) a fitness advantage of *S. invicta* over hybrid in the southern parts of their United States distribution; (2) a fitness advantage of the hybrid over *S. invicta* in the northern parts of their distribution; (3) fitness factors being equal, no competitive advantage for *S. invicta* or the hybrid; and (4) environmental factors being equal, a possible competitive advantage of hybrid over *S. richteri*.

Our behavioral studies demonstrate for the first time hybridization between *S. invicta* and *S. richteri* through a behavioral bioassay and again through a species-specific biochemical character (Vander Meer et al., 1985). This work brings us closer to an understanding of possible mechanisms by which the two fire ant species and their hybrid reached their present distribution in the United States.

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RELEASE KINETICS OF LIQUID FLOWABLE
FORMULATIONS OF GOSSYPLURE, SEX PHEROMONE
OF THE PINK BOLLWORM MOTH *Pectinophora*
gossypiella SAUNDERS (LEPIDOPTERA: GELECHIIDAE)

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Abstract—The kinetics of the release of gossyplure, measured in the laboratory at $34.5 \pm 0.5^\circ\text{C}$, from two liquid flowable formulations are presented and discussed. The data indicate that liquid flowable formulations, defined as those formulations where the active ingredient (in this case gossyplure) is attached to a particulate material, which in turn is suspended in a liquid medium, probably can be successfully used to disseminate pheromones and other behavior-modifying chemicals, although much work remains to be done in the development of these and other liquid flowable formulations.

Key Words—Pheromone formulations, controlled release, release rate determination, release kinetics, liquid flowables, *Pectinophora gossypiella*, Lepidoptera, Gelechiidae.

INTRODUCTION

Despite the fact that sex pheromones have been researched for more than 25 years, their successful commercialization and widespread use in plant protection strategies have not yet been realized. One of the major reasons for this is the fact that the development of efficacious and cost-effective controlled release formulations for behavior-modifying chemicals can be a slow and costly undertaking. The question of the principles of the design of such controlled release

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formulations and their use strategies has recently been reviewed (Weatherston, 1988). Currently there are five basic types of commercial formulation available, namely, micro- and macrocapsules, trilaminates, capillaries, ropes, and liquid flowables. Of these five, the liquid flowables are the most recent development, and interest in this type appears to be wide-ranging, research currently being pursued by Montedison (Italy), Dexter Chem. Intl. (Israel), Monterey Chemical [Mitsubishi] (California), Spray Control Systems (Georgia), and Fermone Inc. (Arizona). A liquid flowable formulation is one in which the pheromone is attached to a particulate material that in turn is suspended in a liquid medium.

Liquid flowable formulations of insect sex pheromones can be used in pest management as disruptants or in the bioirritant strategy. This strategy involves the use, usually as a tank-mix, of a pheromone in conjunction with a compatible registered insecticide. Certain adult female lepidopteran insects (Priesner, 1979), including the pink bollworm (Cook and Shelton, 1978), have been shown to detect their own pheromone, and in some cases (Palanaswamy and Seabrook, 1978; Mitchell et al., 1972; Birch, 1977) behavioral responses have been demonstrated. Taking the pink bollworm *Pectinophora gossypiella* as an example, it is envisaged that increased control over either the insecticide alone or the pheromone alone can be achieved by causing increased in-field movement of adults of both sexes, resulting in increased probability of contacting the insecticide.

The intended use of a pheromone in this manner changes some of the design parameters from those of a disruptant formulation; although it will still be desired to utilize all the pheromone within the field use period, it should be possible to utilize a lesser amount of gossyplure and have a shorter longevity period. It is not intended that the longevity of the pheromone should be much different from the concurrently used pesticide.

At this time, we wish to report preliminary laboratory studies carried out on two liquid flowable formulations of gossyplure obtained in 1984 from Rainbow Chemical Inc., Litchfield, Arizona. The two formulations studied were identical in that both contained the same amount of gossyplure (0.632% active ingredient) [(Z,Z)- and (Z,E)-7,11-hexadecadien-1-yl acetate] impregnated onto a particulate material, which, by the use of emulsifiers and surfactants, was suspended in an aqueous medium. The only difference in the formulations was in the choice of particulate material: one formulation utilized Hi-Sil, an amorphous silica (formulation H), while the other contained perlite, a silicate (formulation P).

METHODS AND MATERIALS

Gas chromatography (GLC) was carried out on a Hewlett-Packard 5790 capillary gas chromatograph fitted with flame ionization detectors and coupled to a Hewlett-Packard 3390A programmable integrator. The column used was 11.5

m × 0.20 mm fused silica, with a stationary phase of methyl silicone gum, operated isothermally at 200°C. The carrier gas was hydrogen, used at a linear velocity of 42 cm/sec. The injector, operated in the split mode (split ratio 100: 1), was held at 240°C with a detector temperature of 300°C. Under these conditions, the isomers are not resolved and gossyplure is seen as one peak with a retention time of 3.50 min.

Determination of the amount of gossyplure in the formulations was carried out by introducing approximately 300 mg of the formulations directly into a tared 5-ml volumetric flask, which was reweighed. About 0.5 ml of a hexane solution of the internal standard (methyl heptadecanoate, 3.77 mg/ml) was then added, and the flask was filled to the 5-ml mark with hexane. A small stirring bar was carefully introduced into the flask, the stopper was inserted, and the contents of the flask were continuously stirred on a magnetic stirrer. At various time intervals, 2- μ l samples were analyzed by GLC.

The determination of the release rate of the gossyplure was carried out by residue analysis. For each formulation, the bottle was well shaken, a sample was drawn up into a Pasteur pipet, and approximately 200 mg was weighed onto a Teflon disk 2 cm in diameter. In this manner, 24 disks were prepared from formulation H (three replicates for eight time periods) and 15 disks from formulation P (three replicates for five time periods). Each disk was placed on a small aluminum foil pan and incubated at $34.5 \pm 0.5^\circ\text{C}$. Three samples from each formulation were analyzed immediately to yield the time-zero amounts of gossyplure. At various time intervals over 10 days, the residues from three randomly chosen disks of each formulation were each washed into a scintillation vial with 2 ml of hexane; 1 ml of the hexane solution of the methyl heptadecanoate was then added, followed a stirring flea and the mixture stirred for 24 hr. After the first day, the formulations had dried and the residual "cake" was eased off the disk into the vial, the disk and the point of the spatula washed with 2 ml of hexane, and the extraction carried out as indicated above. For the GLC analyses, five or six injections were carried out for each disk extract.

Hexane, distilled in glass, was purchased from Burdick and Jackson, Muskegon, Michigan, and used without further purification. Methyl heptadecanoate (99.9%) was obtained from Chromatographic Specialities Ltd., Brockville, Ontario.

RESULTS AND DISCUSSION

Initially it was determined by extraction and subsequent GLC analysis that formulations without the active ingredient yielded no volatile materials and that there was no interference in the area of interest on the chromatograms. The retention times, on methyl silicone, of the technical gossyplure used to prepare the formulations and the material extracted from the formulations were identical.

In order to determine the release rate of the gossypure by residue analysis, the time required to extract all of the gossypure from the formulations had to be ascertained. The results are presented in Figure 1; as can be seen clearly, the gossypure can be extracted from formulation H in a shorter time than from formulation P. Virtually all of the gossypure (95%) has been extracted after 2 hr, whereas only about 65% has been extracted from the perlite formulation. Although there do not appear to any significant differences between the values obtained after 4- and 5-hr extractions and the 24-hr extraction with both formulations, it was determined that for the release rate studies a 24-hr extraction period would be used.

The data presented below are given in micrograms per hour per gram of formulation because liquid flowables are diluted with water or emulsifiable vegetable oil and tank mixed with a compatible registered pesticide when used in the bioirritant strategy.

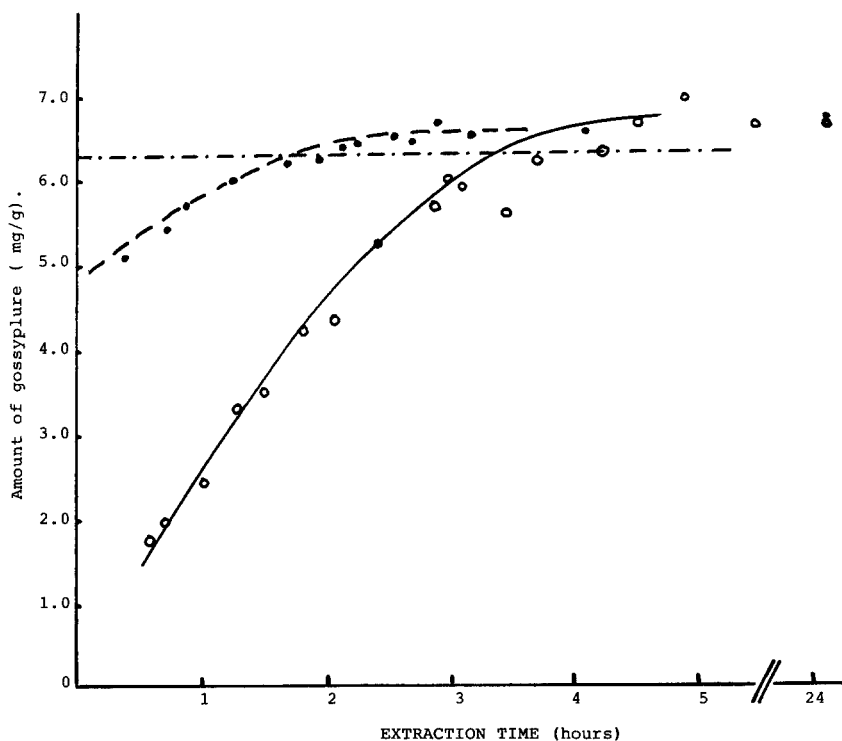


FIG. 1. Residue analysis for gossypure from formulation H [---] and formulation P [—] extracted at ambient temperature for various periods of time. The 24-hr values are the means of five replicated extractions; all other data points are from a representative extraction. The line [- · - · -] represents the stated active ingredient percentage.

Data from the residue analysis of the formulations after various periods of aging at $34.5 \pm 0.5^\circ\text{C}$ are presented in Table 1 and Figure 2. From the table it can be calculated that the mean release rate over 10 days is $19.9 \mu\text{g/hr/g}$ for formulation H and $15.8 \mu\text{g/hr/g}$ for formulation P. Linear regression analysis of these data indicate a correlation coefficient $r^2 = 0.0980$ for both formulations, a mean release rate of $21.1 \mu\text{g/hr/g}$ over 13.6 days for formulation H, and $17.3 \mu\text{g/hr/g}$ over 17 days for Formulation P. Although the regression analyses indicate longevitys of 13.6 and 17.0 days, after 9.5 days in the case of H and 10 days in the case of P there is actually 35.5% and 43.5% of the gossyplure still remaining in the formulation. These results are presented graphically in Figure 2.

Considering formulation H, 64.5% of the gossyplure is emitted in 10 days. As can be seen from the data (Table 1 and Figure 2A), after the first 24 hr, during which the average release is 18.3 mg/hr/g , the rate then remains relatively constant over the next five days at $26.1 \mu\text{g/hr/g}$ ($r^2 = 0.997$) before slowing during the remainder of the 10 days, although there is an unexplained anomalous increase during day 9. The best-fit curve and the regression line (Figure 2A) are diverging at day 10, indicating that the pheromone release from the formulation is slowing significantly. With regard to formulation P, 56.5% of the active ingredient is emitted during 10 days. The data (Table 1 and Figure 2B) indicate that after the first 49 hr, during which there is a low mean release of $6 \mu\text{g/hr/g}$, the release kinetics are zero order over the next six days ($r^2 = 0.999$) at a release of $21.2 \mu\text{g/hr/g}$. During the ninth and tenth days, the rate has slowed to less than $10 \mu\text{g/hr/g}$; the best-fit curve and regression line (Figure 2B) are beginning to diverge at this time.

Both formulations exhibit a latency period, which we believe to be due to a slower release of gossyplure until all of the water has evaporated. This period

TABLE 1. RESIDUE ANALYSIS FOR GOSSYPLURE IN FORMULATIONS H AND P AGED AT $34.5 \pm 0.5^\circ\text{C}$ FOR UP TO 10 DAYS^a

Formulation	Time (hr)										
	0	24	48	49	72	96	144	168	192	226	240
Formulation H											
Gossyplure (mg/g)	6.96	6.52	5.86		5.11		3.38	3.17	2.77	2.47	
±SD	0.09	0.06	0.10		0.18		0.09	0.13	0.18	0.07	
Formulation P											
Gossyplure (mg/g)	6.71			6.42		5.38	4.38		3.38		2.92
±SD	0.01			0.03		0.05	0.03		0.06		0.17

^aLinear regression: Formulation H— $r^2 = 0.980$; $a = 6.836$; $b = -0.0211$. Formulation P— $r^2 = 0.980$; $a = 6.947$; $b = -0.0173$.

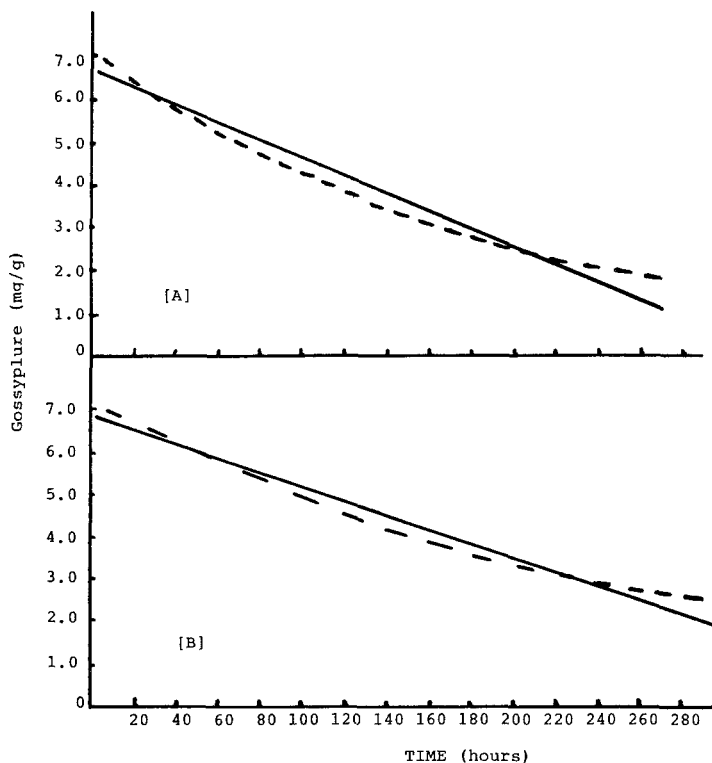


FIG. 2. Residue analyses for gossyplure from (A) formulation H and (B) formulation P, aged at $34.5 \pm 0.5^\circ\text{C}$ for up to 10 days. [---] best-fit curve from observed data; [—] linear regression.

is probably only a few hours in duration, but the design of the experimental protocol precluded confirmation. It is important to know the distribution of the gossyplure throughout the formulation, especially in regard to whether it migrates to and/or is released from the liquid phase. Studies have indicated (Weatherston and Miller, unpublished) that, of the gossyplure recovered from formulation H, 81% was associated with the Hi-Sil, while 19% was associated with the oils and emulsifiers. No pheromone was found in the aqueous phase.

The above data indicate that the formulations tested could be used as a basis for a development program involving both laboratory and field studies to define the effects on rate release and efficacy of solid-phase loading, gossyplure loading, various emulsifiers, nonreversible adsorption of the active ingredient onto the particle, temperature, etc. Preliminary indications are that formulations such as those tested could possibly, in the future, become commercial products used in a bioirritant strategy of plant protection.

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INFLUENCE OF PHEROMONE TRAP COLOR AND DESIGN ON CAPTURE OF MALE VELVETBEAN CATERPILLAR AND FALL ARMYWORM MOTHS (LEPIDOPTERA: NOCTUIDAE)¹

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Abstract—Three pheromone traps were evaluated in paired field trials for effectiveness in capturing wild male velvetbean caterpillar moths (VBC), *Anticarsia gemmatalis* Hübner, and fall armyworm moths (FAW), *Spodoptera frugiperda* (J.E. Smith), using a wind-oriented trapping device. The traps were: (1) the standard multicolored bucket trap consisting of a forest green canopy, yellow funnel, white bucket and open (i.e., single wire) pheromone holder; (2) a forest green monocolored bucket trap; and (3) the Multi-Pher-1 trap consisting of a blue-green canopy, white funnel, white bucket, and white, multislotting pheromone holder. The Multi-Pher-1 trap differs primarily from the other two in that its entrance is smaller in diameter and is partially obstructed by the pheromone holder. Significantly fewer VBC and FAW males were captured in pheromone-baited monocolored (forest green) bucket traps than standard multicolored bucket traps. The Multi-Pher-1 trap also caught significantly fewer VBC moths than the standard multicolored bucket trap, but there was no significant difference in numbers of FAW moths caught in the two traps. The results further demonstrate the importance of considering visual cues, in this case color, in the design of pheromone traps for nocturnal insects.

Key Words—Trap color, trap design, velvetbean caterpillar, *Anticarsia gemmatalis*, fall armyworm moth, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, electroretinogram.

¹This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by USDA.

INTRODUCTION

International Pheromones Moth Traps (bucket trap; International Pheromone Systems, Merseyside, England) baited with pheromone are used widely to survey adult populations of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Mitchell et al., 1985). Mitchell and Heath (1986) also demonstrated the utility of bucket traps for surveying adult populations of the velvetbean caterpillar (VBC), *Anticarsia gemmatalis* Hübner, with sex pheromone. In the course of these and subsequent investigations involving pheromones of the FAW, VBC, and *Heliothis* spp., we observed a propensity for bumblebees, *Bombus* sp., to be captured in bucket traps along with the target species. Captures of bumblebees were most common in late summer and fall when the traps were deployed in weedy areas with plants having large conspicuous blooms. We attributed the apparent attraction of bumblebees to the bucket trap's colorful appearance—a protective canopy of forest green, an entrance funnel of bright yellow, and a white bucket receptacle. Bucket traps of identical dimensions also are available with one color throughout—forest green. The present study was designed to determine if the forest green bucket trap could be used in place of the multicolored bucket trap without sustaining significant reductions in captures of male FAW or VBC moths. We also evaluated the Multi-Pher-1 (Bio-Controle Services, Ste-Foy, Canada), a trap somewhat similar in design to the bucket trap but costing approximately 30% less. This trap has a blue-green canopy, white funnel, white bucket, and white, multislotting pheromone holder. The design of the Multi-Pher-1 trap differs from the standard multicolored bucket trap in that the entrance is smaller, the funnel is abbreviated, and the funnel opening is partially obstructed by the pheromone holder.

METHODS AND MATERIALS

Field Trials. All tests were conducted in northwest Alachua County, Florida, in fall 1987. Pheromone baits for the FAW were prepared by Terochem Laboratories, Ltd. (Alberta, Canada). The four-component pheromone blend formulated on rubber septa at 2.0 mg/dispenser consisted of (percentage by weight): (*Z*)-7-dodecen-1-ol acetate, 0.45%; (*Z*)-9-dodecen-1-ol acetate, 0.25%; (*Z*)-9-tetradecen-1-ol acetate, 81.61%; and (*Z*)-11-hexadecen-1-ol acetate, 17.69%.

The pheromone used in tests with the VBC was prepared in our laboratory. The pheromone was purified by AgNO₃ high-performance liquid chromatography (Heath and Sonnet, 1980). Analysis of the purified pheromone was obtained on a capillary gas chromatograph (Varian 3700), and the columns used for analysis were 50 m × 0.25 mm ID methyl silicone (OV-1); 50 m × 0.25 mm ID Carbowax 20 M; and 42 m × 0.27 mm ID cholesteryl-*p*-chlorocinna-

mate, a liquid crystal phase described by Heath et al. (1979). Analysis of the pheromone with these columns showed the material to be at least 99% pure. Before loading, the rubber septa (5 × 9 mm; No. 8753-D22, A.H. Thomas, Philadelphia, Pennsylvania) were extracted with CH₂Cl₂ for 24 hr and air dried. A septum was loaded by depositing 0.5 mg of a blend consisting of (Z,Z,Z)-3,6,9-eicosatriene and (Z,Z,Z)-3,6,9-heneicosatriene (40 and 60%, respectively) dissolved in 100 μl hexane into the end with the largest reservoir.

Wind-oriented traps (WORT) (Mitchell et al., 1988) were used to make simultaneous comparisons of the bucket trap combinations (standard multicolored vs. forest green, Figure 1a) or the standard multicolored bucket trap and the Multi-Pher 1 trap (Figure 1b). The WORT consists of three major compo-

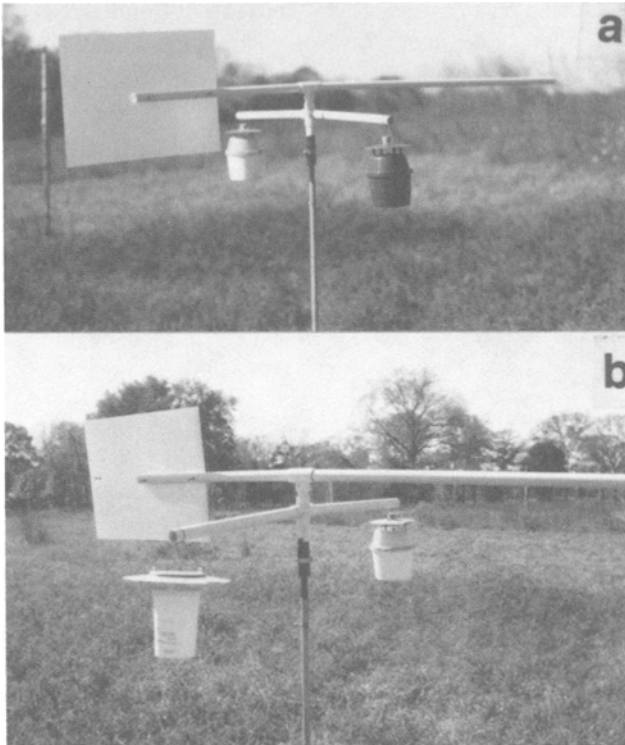


FIG. 1. Wind-oriented trapping device (WORT) used to determine the effect of color (a) and design (b) on capture of male velvetbean caterpillar and fall armyworm moths in traps baited with sex pheromone. (a) International Pheromones bucket traps (BT) mounted on WORT (left, standard BT, with forest green top, yellow funnel, and white bucket; BT on right is forest green throughout). (b) Standard BT on right and Multi-Pher-1 trap on left.

nents: tail and nose assembly, pivot, and support arms arranged perpendicularly to the tail and nose sections. The design of the WORT is such that treatments are always aligned in the same relationship relative to the prevailing wind, thereby minimizing the possibility of interactions among closely spaced pheromone traps caused by shifts in surface winds. The test traps were positioned 1 m apart opposite each other on the WORT support arms. WORTs were positioned so that the bottom of each trap bucket was ca. 1 m from the soil surface. The bucket receptacle of each trap contained a small piece of dichlorovos (Vapona) insecticide strip that served as a killing agent for trapped moths.

Three WORTs baited with VBC pheromone and three WORTs baited with FAW pheromone were operated simultaneously in a soybean field (> 35 hectares) that also had a high population of young (pretassel) volunteer corn plants. The WORTs were spaced 300–500 m apart throughout the field. In the first test with the VBC, the standard multicolored bucket trap (forest green canopy, yellow funnel, white bucket, Figure 2a) was compared to the monocolored forest green bucket trap (not shown). The first trial with the FAW involved the same trap color combinations on different WORTs and was carried out simulta-

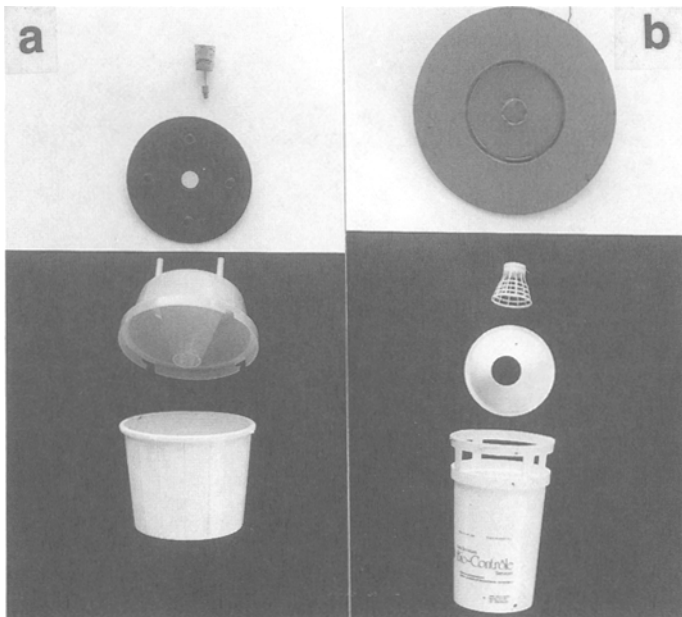


FIG. 2. Trap components: (a) multicolored bucket trap with forest green canopy, yellow funnel, white bucket; (b) Multi-Pher-1 trap with blue-green canopy, white pheromone holder, white funnel and bucket.

neously with the first VBC trap test. The second test for both the VBC and FAW compared the standard bucket trap with the Multi-Pher-1 trap (blue-green canopy, white multislotted pheromone holder, white funnel, white bucket) (Figure 2b). These trials also were carried out simultaneously. In all cases, the trap colors were an integral part of each molded component, i.e., the colors were not simply painted onto the component's exterior surfaces. Moth catches were recorded daily, and the treatments (i.e., traps) were rotated between positions on each WORT. Pheromone baits were replaced every two weeks. Each collection was considered a replicate. Differences between means were separated using the *t* test for paired treatments (Steel and Torrie, 1960).

Spectral Analysis of Compound Eye. Spectral sensitivity of the compound eye of male VBC and FAW moths (six of each) to monochromatic light at wavelengths from 350 to 675 nm was measured electrophysiologically. The moths were from cultures reared in our laboratory on a modified pinto bean diet (Guy et al., 1985). The moths were 2–3 days old when tested. The moths were immobilized on a wax base to facilitate the insertion of microelectrodes into their compound eyes. A recording stainless-steel microelectrode was inserted 20–30 μm into the mid-dorsal region of one eye that was positioned with the mid-lateral center directed toward the stimulating light. The indifferent electrode was positioned in a similar position in the unilluminated eye. A light beam from a monochromator was focused directly on the lateral surface of the eye and head of the moth. The moths were dark-adapted for 45–60 min until they were at maximum sensitivity in a light-proof electrically shielded cage.

The electroretinogram techniques were similar to those described by Agee (1973) and Agee and Patterson (1983). Light pulses of 200 msec were delivered at a rate of one per 2 sec and intensity adjusted to produce a "criterion" electrical response of 200 μV in the eye per stimulus. The light pulse duration was controlled by an electrically driven solenoid shutter. Monochromatic light was produced by a quartz-halogen lamp with intensity adjusted to the criterion response by quartz-icnel neutral density wedge and filters. The intensity of light stimuli was determined at all test wavelengths with a calibrated thermopile (accuracy traceable to National Bureau of Standards) and a nanovolt meter in microwatts per square centimeter. Test wavelengths from 350 to 675 nm were selected with a grating monochromator. The electrical responses generated by the photoreceptors in the eye were amplified with a biological amplifier and displayed on an oscilloscope for analysis.

Spectral Reflectance of Trap Colors. The spectral reflectance of samples (2.5 cm \times 2.5 cm) of the various colored trap components were determined using a Varian 634S recording reflectance spectrophotometer. The surfaces were scanned at wavelengths from 330 to 850 nm with the monochromator slit width set at 1 mm and operated at a scan rate of 50 nm/min to obtain maximum accuracy. Eastman Kodak white (No. 6091) was the 100% standard.

RESULTS AND DISCUSSION

Color had a profound effect on pheromone trap effectiveness in capturing male VBC moths (Figure 3A). The standard multicolored bucket trap captured 6.7 times more VBC moths than the forest green bucket trap (difference significant at $P < 0.01$, paired t test). The standard multicolored bucket trap also captured significantly more male FAW moths than the forest green bucket trap (Figure 4A; $P < 0.01$), although the magnitude of increase was not as great as for the VBC.

The standard multicolored bucket trap captured significantly more ($P < 0.01$; Figure 3B) male VBC moths than the Multi-Pher-1 trap. However, there was no significant difference in the numbers of FAW males captured in the standard multicolored bucket trap and the Multi-Pher-1 trap (Figure 4B).

The spectral reflectance of the standard multicolored bucket trap parts showed that white had 91–100% reflection at wavelengths from 420 to 680 nm. The yellow reflected 55% of the incident light at wavelengths from 530 to 680 nm. The forest green had a reflectance peak at 510–530 nm of only 16% of the incident light. By contrast the blue–green canopy of the Multi-Pher-1 trap reflected 43% of the incident light at wavelengths of 480–510 nm (Figure 5).

The eyes of both the VBC and FAW were most sensitive at two similar peaks: a broad major peak in the green–yellow range from 480 to 570 nm (Fig-

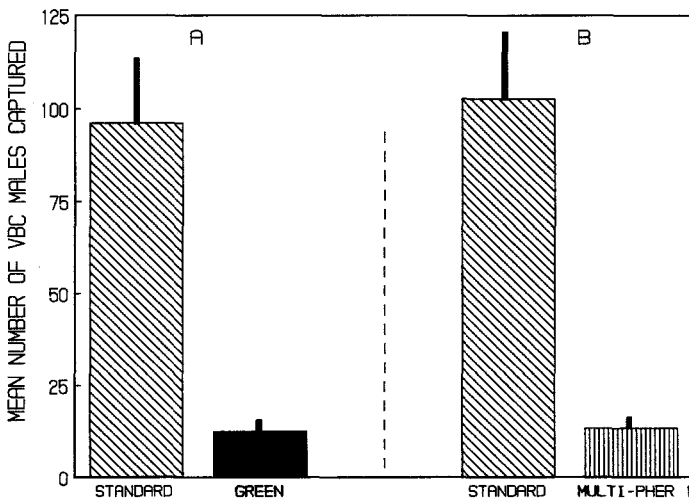


FIG. 3. Capture of male velvetbean caterpillar moths in: (A) standard bucket trap (BT) versus the all-green BT trap (33 replications); (B) standard BT versus the Multi-Pher-1 trap (21 replications). Difference between means within A and B were significant, $P < 0.01$, paired t test. Thin black bars represent standard error of the mean.

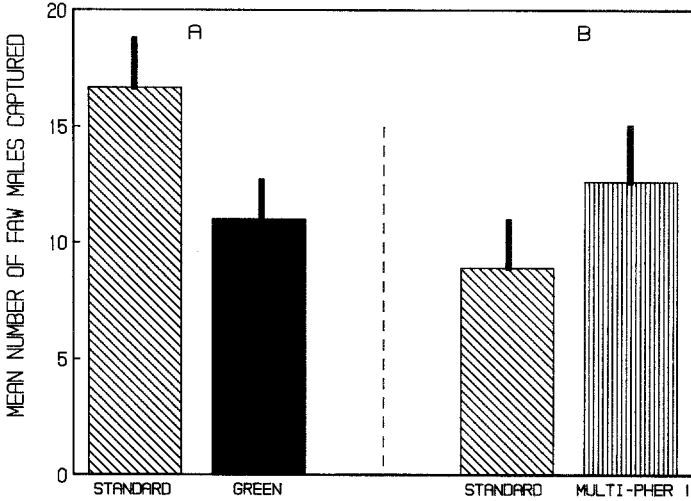


FIG. 4. Capture of male fall armyworm moths in: (A) standard bucket trap (BT) versus the all-green BT (43 replications); (B) standard BT versus Multi-Pher-1 trap (22 replications). Difference in mean moth captured within A was significant, $P < 0.01$, paired t test; difference in mean moth captured within B was not significant. Thin black bars represent standard error of the mean.

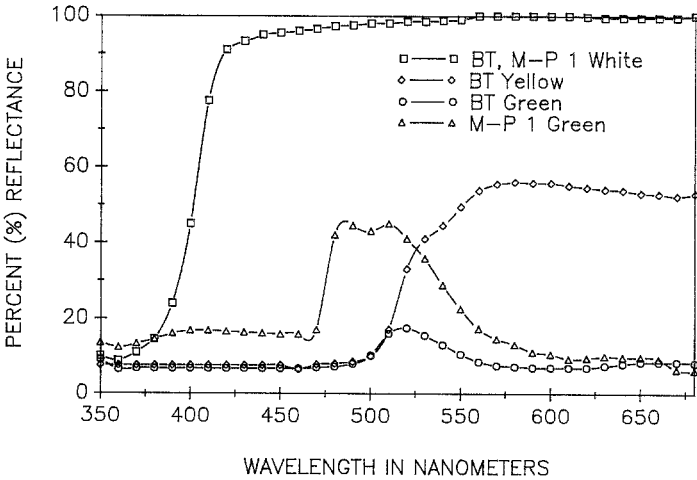


FIG. 5. Spectral reflectance of components of the multicolored bucket trap (BT), forest green BT, and Multi-Pher-1 trap (M-P 1) at wavelengths from 350 to 680 nm.

ure 6). The FAW was most sensitive at 530 nm while the VBC was most sensitive at 520 nm. Both species had smaller second peaks centered in the ultraviolet at 365 nm. The spectral sensitivity curve for both species were quite similar to sensitivity curves reported for the corn earworm moth, *H. zea* (Boddie), and the tobacco budworm moth, *H. virescens* (F.) (Agee, 1973).

No attempt was made to quantify the effect of individual colors, except forest green, and then only in the case of the monocolored forest green trap. The impact of the standard multicolored trap's forest green canopy on trap catch is unknown. However, because of the canopy's small surface area—relative to the surface area of the funnel and bucket—and horizontal orientation, it is considered unlikely that the standard multicolored trap's forest green canopy had much, if any, impact on moth catch.

Mitchell et al. (1985, 1988) reported that standard multicolored bucket traps baited with pheromone captured significant numbers of male VBC and FAW moths, whereas unbaited control traps caught few or no moths. Thus, it does not appear that the multicolored bucket trap per se is attractive to either the VBC or FAW. Based upon the spectral reflectance of the various colored trap components and the spectral sensitivity of the compound eyes of the two species, it appears that the factor responsible for the decreased capture of VBC moths—and to a lesser extent FAW moths—was the forest green color of the monocolored bucket trap, which showed the lowest light reflectance. Thus, it

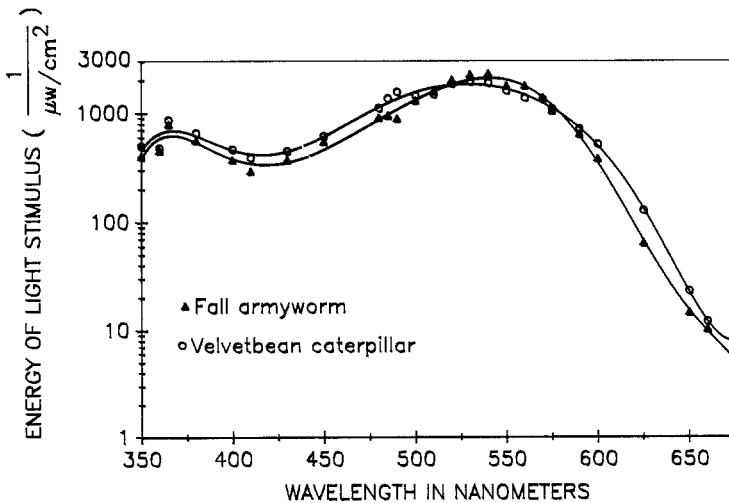


FIG. 6. Mean spectral sensitivity of the compound eyes of six laboratory-reared velvetbean caterpillar moths and six fall armyworm moths at wavelengths from 350 to 675 nm.

appears that visual stimuli tended to overcome a normally strong attraction response to a powerful olfactory stimulant—sex pheromone.

Failure of the Multi-Pher-1 trap to catch significant numbers of VBC also may have been due to visual stimuli associated with the white trap (Figure 2B), which had a reflectance in the range of 91–100%. However, the reduced catch of VBC moths with the Multi-Pher-1 trap probably was due more to the physical design of the trap itself rather than light reflectance. The Multi-Pher trap's funnel entrance was partially obstructed by the pheromone holder protruding downward from the canopy. Previous observations in field situations on the response of both the VBC and FAW to synthetic pheromone dispensed from rubber septa in multicolored bucket traps revealed that attracted moths generally flew rapidly around, above, and below the septa in uneven arcs; they were captured after striking the side of the funnel and tumbling into the bucket. The VBC is a much larger moth than the FAW. Presumably then, the pheromone holder for the Multi-Pher-1 trap kept the larger VBC moths from falling into the trap receptacle.

The results reported here contrast sharply with those of McLaughlin et al. (1975), which showed that pheromone traps with low spectral reflectance in the 360- and 550-nm regions (e.g., black) were more effective in capturing the nocturnal cabbage looper, *Trichoplusia ni* (Hübner), and soybean looper, *Pseudoplusia includens* (Walker), than brightly colored traps (e.g., daylight fluorescent yellow). Nevertheless, both studies demonstrate the importance of considering the visual response of night-flying insects in the design of pheromone traps. While the correct choice of color probably will not overcome a trap design that fails to address adequately basic parameters that in themselves may limit effectiveness (e.g., too small an entrance), the wrong choice of color could render an otherwise well-designed trap virtually useless. Furthermore, the results reported here support previous studies that showed the standard multicolored bucket trap to be an effective tool for surveying adult populations of the velvetbean caterpillar (Mitchell and Heath, 1986) and fall armyworm (Mitchell et al., 1985) using synthetic sex pheromone.

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INHIBITION AND PROMOTION OF GERMINATION BY SEVERAL SESQUITERPENES

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Abstract—The sesquiterpene lactones, burrodin, confertiflorin, desacetylconfertiflorin, dihydroparthenolide, parthenin, and 7 α -hydroxy-3-desoxyzaluzanin C, and the sesquiterpene ester guayulin A were assayed at concentrations of 1, 10, and 100 μ M for effects on seed germination of 16 dicot and nine monocot species. Six of the dicot and two of the monocot species were affected by one or more of these compounds. Germination was both inhibited and promoted, depending on the compound and the specific species or cultivars, at concentrations as low as 1 μ M. For example, guayulin A, which promoted the germination of lettuce at all concentrations tested, inhibited the germination of tomato. Confertiflorin stimulated germination of the lettuce cultivar Grand Rapids at 1 μ M, but inhibited germination of a light-sensitive cultivar at all concentrations tested.

Key Words—Burrodin, confertiflorin, desacetylconfertiflorin, dihydroparthenolide, parthenin, 7 α -hydroxy-3-desoxyzaluzanin C, guayulin A, sesquiterpene lactone, allelopathy, seed germination.

INTRODUCTION

Over 3000 sesquiterpene lactones have been chemically characterized (Vasquez and Fischer, unpublished), but relatively little work has been done on the biological activity and ecological significance of these compounds (Picman, 1986; Duke, 1986). Several studies have suggested that sesquiterpene lactones may function as plant growth regulators. Reported allelopathic effects include both

stimulation and inhibition of radical growth and inhibition of germination (McCahon et al., 1973; Spencer et al., 1984; Stevens and Merrill, 1985). Sesquiterpene lactones have also been implicated as allelopathic agents of *Parthenium hysterophorus* L. (Kanchan and Jayachandra, 1980) and *Ambrosia cumanensis* H.B.K. (del Amo and Anaya, 1978). However, in preliminary studies of 10 sesquiterpene lactones, including four of those examined in the present study, Fischer and Quijano (1985) found only minor effects when these compounds were screened at single concentrations (50–100 μM) on the germination of 12 cultivated and weedy species.

The objective of this study was to examine the effect of the six sesquiterpene lactones, burrodin, confertiflorin, desacetylconfertiflorin, dihydroparthenolide, parthenin, and 7α -hydroxy-3-desoxyzyluzanin C, and the sesquiterpene ester, guayulin A, (Figure 1) on the germination of 25 plant species at concentrations of 1, 10, and 100 μM .

METHODS AND MATERIALS

Isolation of Sesquiterpene Lactones. The isolation of the sesquiterpene lactones used in these experiments has previously been described. Burrodin was isolated from *Ambrosia dumosa* (Gray) Payne (Seaman, 1976), confertiflorin and desacetylconfertiflorin from *Ambrosia confertiflora* DC. (Fischer and Mabry, 1967), 11,13-dihydroparthenolide from *Ambrosia artemisiifolia* L. (Fischer et al., 1981), and 7α -hydroxy-3-desoxyzyluzanin C from *Podochaenium eminens* (Lagasca) Schultz-Bip. (Fronczek et al., 1984). Parthenin was isolated from *Parthenium hysterophorus* L. and guayulin A from *Parthenium argentatum* Gray by normal procedures for sesquiterpenes (Fischer et al., 1975).

Multiseed Germination Bioassay. The assay seeds, common names, seed sources, and number of seeds per replicate are shown in Table 1. All undersized and damaged seeds were discarded, and the assay seeds were preselected for uniformity of size and seed coat color in the case of weed species. Assays were performed using 9-cm plastic Petri dishes lined with filter paper. The multiseed assay protocol was the same 25°C, 72 hr, dark assay with eight replicates described by Bradow (1985), including the presence of 0.1% dimethylsulfoxide (DMSO) added as an initial solubilizing agent to all test solutions and parallel deionized water controls. Test solutions (3 ml) contained 1, 10, or 100 μM of the appropriate sesquiterpene. Where necessary, test solution pH was adjusted to 6.8 with 0.1 N KOH, and the osmolarities of all test solutions (0–5 mosm) were below levels (≥ 18 mosm) inhibitory to the most sensitive test seeds, i.e., tomato, lettuce, and carrot (data not shown).

Statistical Analyses. Using a completely random design with eight replications, the count data were normalized by the transformation $(x + 0.5)^{0.5}$. Data

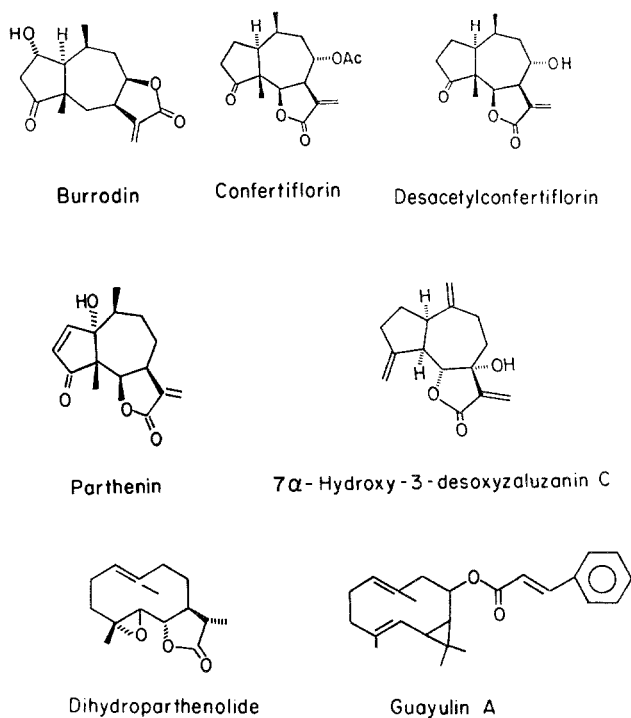


FIG. 1. Structures of the sesquiterpenes used in this study.

for each seed species were analyzed separately, and significant differences between compound effects were determined using one-way analyses of variance and the Waller-Duncan Bayesian k ratio t test (significance level, $P = 0.01$) (Steel and Torrie, 1980). Concentration effects were examined via two-way analyses of variance (significance level, $P = 0.01$) (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

The germination of the six dicot species listed in Table 2 was affected by one or more of the six sesquiterpene lactones, burrodin, confertiflorin, desacetylconfertiflorin, dihydroparthenolide, parthenin, and 7 α -hydroxy-3-desoxyzaluzanin C, and the ester, guayulin A. The seeds of the 10 dicot species listed in Table 1, but not in Table 2, were not significantly affected. Compound differences and the compound \times concentration interactions were significant ($P = 0.01$) in each of the two-way analyses of variance for the individual sensitive seed species.

TABLE 1. SPECIES AND COMMON NAMES, NUMBER OF SEEDS PER REPLICATE AND SOURCES OF SEEDS USED IN MULTISEED BIOASSAYS

Species	Common Name	Seeds per replicate	Source ^a
Dicots			
<i>Amaranthus palmeri</i> S. Wats	Palmer amaranth	25	4
<i>Amaranthus retroflexus</i> L.	Redroot pigweed	25	5
<i>Brassica napus</i> L.	Rape	20	5
<i>Capsella bursa-pastoris</i> (L.) Medik	Shepherdspurse	25	5
<i>Chenopodium album</i> L.	Common lambsquarters	25	5
<i>Cucumis sativus</i> L. cv. Marketmore	Cucumber	10	2
<i>Daucus carota</i> L. cv. Danvers Half Long	Carrot	20	2
<i>Lactuca sativa</i> L. cv. Grand Rapids	Lettuce	20	2
Light insensitive	Lettuce	20	3
Light sensitive	Lettuce	20	3
<i>Lepidium sativum</i> L.	Curly cress	20	2
<i>Linum usitatissimum</i> L.	Flax	20	3
<i>Lycopersicon esculentum</i> Mill. cv. Homestead	Tomato	20	2
<i>Medicago sativa</i> L.	Alfalfa	20	5
<i>Portulaca oleracea</i> L.	Common purslane	25	5
<i>Rumex acetosella</i> L.	Red sorrel	25	5
<i>Sida spinosa</i> L.	Prickly sida/teaweed	20	5
<i>Trifolium incarnatum</i> L.	Crimson clover	20	3
Monocots			
<i>Allium cepa</i> L. cv. Texas Early Grano 501	Onion	20	1
<i>Avena sativa</i> L.	Oats	20	3
<i>Bromus inermis</i> Leyss.	Smooth brome	20	5
<i>Bromus secalinus</i> L.	Cheat	20	5
<i>Echinochloa crus-galli</i> (L.) Beauv.	Barnyard grass	20	5
<i>Eragrostis curvula</i> (Shrad.) Nees	Lovegrass	20	5
<i>Lolium</i> spp.	Ryegrass	20	3
<i>Setaria italica</i> (L.) Beauv.	Foxtail millet	20	5
<i>Sorghum bicolor</i> (L.) Moench	Sorghum	15	3

^a 1: Baxter's, Weslaco, Texas 78596; 2: Burpee, Warminster, Pennsylvania 18974; 3: Carolina Biological Supply, North Carolina 27215; 4: R. Menges, USDA, Weslaco, Texas 78596; 5: Valley Seed Service, Fresno, California 93791.

All of the sesquiterpenes affected seed germination in more than one of the listed species, both promoting and inhibiting (or delaying) germination. Desacetylconferitiflorin, parthenin, and 7 α -hydroxy-3-desoxyzaluzanin C each affected five of the eight sensitive species, and the three lettuce cultivars were sensitive to all seven sesquiterpenes. The effects of the sesquiterpenes on lettuce

germination varied with concentration and cultivar. All three guayulin A concentrations tested increased germination of all three lettuce cultivars. Confer-tiflorin stimulated germination of lettuce cultivar Grand Rapids at 1 μM , but inhibited germination of the light-sensitive cultivar at all concentrations tested. Tomato seed germination was increased by 1, 10, and 100 μM burrodin and parthenin and by 1 and 10 μM desacetylconfer-tiflorin, dihydroparthenolide, and 7 α -hydroxy-3-desoxyzaluzanin C. All levels of guayulin A tested and 100 μM 7 α -hydroxy-3-desoxyzaluzanin C inhibited tomato germination.

The seeds of *Amaranthus palmeri* and *A. retroflexus* germinated minimally under the bioassay conditions used. Under more permissive conditions, i.e., alternating 20/30°C for three days, control samples from the same seed lots germinated 89.6% (*A. palmeri*) and 70.5% (*A. retroflexus*), respectively (Bradow et al., 1988). Several seed lots of these weedy amaranth species exhibited an environmentally induced dormancy that fluctuated in degree over time (5–31% germination), while during the same time period germination under promotive environmental conditions remained in the range of $90 \pm 2\%$ for *A. palmeri* and $70 \pm 3\%$ for *A. retroflexus* (data not shown). This environmentally induced dormancy was alleviated by several synthetic analogs of the sesquiterpene lactone strigol (Bradow et al., 1988), but not by strigol itself or any of the sesquiterpenes considered in this paper.

Only two monocot species were significantly affected by one or more of these sesquiterpenes (Table 3). Again, the seven monocot species listed in Table 1 and not appearing in Table 3 were insensitive to the test compounds. Barnyard-grass was inhibited only by 100 μM parthenin. In the two-way analyses of variance of the sorghum data, compound, concentration, and the compound \times concentration interaction were all significant ($P = 0.001$). Sorghum germination was inhibited by 1, 10, and 100 μM burrodin, parthenin, and desacetyl-confer-tiflorin, and by 10 and 100 μM confer-tiflorin. Sorghum germination was increased by 1, 10, and 100 μM dihydroparthenolide and 7 α -hydroxy-3-desoxy-zaluzanin C.

In a few cases, apparently aberrant results were noted; for example, parthenin did not affect *Sida spinosa* germination at 100 and 10 μM , but a significant reduction was observed at 1 μM (Table 2). This study involved 594 individual treatments (7 compounds \times 3 concentrations \times 27 species and cultivars plus controls) and, at the 0.01 level of probability used for statistical significance, a few instances in which a value is falsely found significantly different from the control are expected (type I errors).

In summary, the data demonstrate that sesquiterpenes can selectively promote or inhibit germination at concentrations as low as 1 μM . The activity of these compounds is considerably higher than the activity of many allelochemicals reported to affect seed germination (cf. Duke, 1986). A direct extrapolation of these results to field situations cannot be made. However, it is not

TABLE 2. RESPONSES OF DICOT SEEDS TO AQUEOUS SOLUTIONS OF SESQUITERPENES

Seed	Compound	Germination (%) at varying concentrations		
		100 μ M	10 μ M	1 μ M
<i>Amaranthus palmeri</i>	Burrodin	11.4 bc	18.7 a	17.4 ab
	Confertiflorin	7.8 c	17.8 a	13.2 bc
	Desacetylconfertiflorin	15.1 ab	22.1 a	25.4 a
	Dihydroparthenolide	13.2 abc	14.0 ab	8.6 c
	Parthenin	17.6 ab	9.5 bc	9.1 c
	7-OH-3-desoxyzalizanin C	0.8 d	6.7 c	11.4 bc
	Guayulin A	12.8 bc	10.2 bc	9.4 c
	Control	15.2 ab	15.2 ab	15.2 bc
<i>Amaranthus retroflexus</i>	Burrodin	3.0 a	5.2 bcd	5.2 a
	Confertiflorin	4.1 a	3.8 cde	4.6 a
	Desacetylconfertiflorin	2.5 a	14.5 a	4.2 a
	Dihydroparthenolide	4.8 a	1.4 e	4.0 a
	Parthenin	4.4 a	8.8 ab	4.0 a
	7-OH-3-desoxyzalizanin C	1.7 a	3.6 cde	3.3 a
	Guayulin A	4.1 a	1.6 de	2.8 a
	Control	4.8 a	4.8 cde	4.8 a
<i>Daucus carota</i>	Burrodin	51.0 a	44.6 ab	44.2 a
	Confertiflorin	45.2 ab	53.1 a	46.8 a
	Desacetylconfertiflorin	53.2 a	41.2 ab	48.1 a
	Dihydroparthenolide	52.3 a	37.1 b	47.3 a
	Parthenin	37.6 b	51.0 ab	48.5 a
	7-OH-3-desoxyzalizanin C	16.9 c	52.3 ab	44.4 a
	Guayulin A	44.7 ab	46.6 ab	52.7 a
	Control	49.4 a	49.4 ab	49.4 a
<i>Lactuca sativa</i> (cv. Grand Rapids)	Burrodin	58.5 de	70.4 bc	68.6 ef
	Confertiflorin	61.3 cd	60.9 c	86.2 a
	Desacetylconfertiflorin	83.6 a	86.1 a	76.7 bcd
	Dihydroparthenolide	48.6 e	45.9 d	47.3 g
	Parthenin	53.3 de	68.2 c	73.3 cde
	7-OH-3-desoxyzalizanin C	38.2 f	63.0 c	63.4 f
	Guayulin A	83.1 a	82.9 a	79.8 abc
	Control	70.6 bc	70.6 bc	70.6 de
<i>Lactuca sativa</i> (light insensitive)	Burrodin	31.6 ab	24.0 cd	22.8 cd
	Confertiflorin	7.6 c	48.4 a	15.6 de
	Desacetylconfertiflorin	10.8 c	24.6 cd	5.4 f
	Dihydroparthenolide	37.3 a	13.2 d	9.8 ef
	Parthenin	13.6 c	17.0 d	8.0 f
	7-OH-3-desoxyzalizanin C	11.8 c	28.1 c	33.6 ab
	Guayulin A	39.7 a	41.8 ab	39.6 a
	Control	23.5 b	23.5 cd	23.5 bcd

TABLE 2. Continued

Seed	Compound	Germination (%) at varying concentrations		
		100 μ M	10 μ M	1 μ M
<i>Lactuca sativa</i> (light sensitive)	Burrodin	2.0 c	1.0 de	16.2 c
	Confertiflorin	5.2 c	5.7 c	8.9 d
	Desacetylconfertiflorin	2.3 c	4.2 cd	3.8 e
	Dihydroparthenolide	3.6 c	0.0 e	2.4 e
	Parthenin	4.6 c	5.0 cd	20.3 c
	7-OH-3-desoxyzalizanin C	6.6 c	21.0 b	30.2 b
	Guayulin A	47.3 a	69.5 a	69.1 a
	Control	19.8 b	19.8 b	19.8 c
	<i>Lycopersicon esculentum</i>	Burrodin	94.3 a	89.2 a
Confertiflorin		89.2 ab	92.4 ab	88.0 ab
Desacetylconfertiflorin		84.7 bc	89.2 a	95.0 a
Dihydroparthenolide		77.8 c	82.8 a	91.2 a
Parthenin		95.6 a	92.4 a	89.3 a
7-OH-3-desoxyzalizanin C		4.4 e	86.7 a	89.9 a
Guayulin A		28.8 d	44.8 d	41.8 d
Control		80.7 bc	80.7 bc	80.7 bc
<i>Sida spinosa</i>		Burrodin	25.8 ab	39.0 ab
	Confertiflorin	32.1 a	45.0 ab	41.3 b
	Desacetylconfertiflorin	28.8 ab	47.0 ab	30.8 de
	Dihydroparthenolide	31.7 a	66.4 a	33.6 bcd
	Parthenin	30.7 a	43.1 ab	15.7 f
	7-OH-3-desoxyzalizanin C	29.0 ab	30.9 ab	13.6 f
	Guayulin A	18.0 b	25.4 b	39.8 b
	Control	32.1 a	32.1 ab	32.1 cd

^aPercent germination data are means of eight replications. Values in a column associated with a given seed and concentration and followed by the same letter(s) are not significantly different according to the Waller-Duncan Bayesian k ratio ($k = 100$) t test.

unreasonable to hypothesize that localized concentrations of sesquiterpenes in soil might reach the low concentrations used in this study as a result of root exudation or litter decomposition. Thus, these results support the hypothesis (Muller, 1969) that allelochemicals can play a significant role in vegetation patterning and that microscale variations in the soil chemical environment, such as those caused by breakdown of plant litter and release of secondary metabolites, are important in the germination and establishment of individual seeds.

TABLE 3. RESPONSES OF MONOCOT SEEDS TO AQUEOUS SOLUTIONS OF SESQUITERPENES

Seed	Compound	Germination (%) at varying concentrations		
		100 μ M	10 μ M	1 μ M
<i>Echinochloa crus-galli</i>	Burrodin	88.0 a	86.5 a	89.3 ab
	Confertiflorin	88.1 a	86.8 a	86.8 ab
	Desacetylconfertiflorin	88.0 a	94.3 a	86.8 ab
	Dihydroparthenolide	90.6 a	89.9 a	85.4 ab
	Parthenin	79.9 b	89.2 a	91.2 a
	7-OH-3-desoxyzaluzeanin C	89.2 a	90.6 a	87.4 ab
	Guayulin A	88.6 a	88.0 a	80.4 b
	Control	87.4 a	87.4 a	87.4 ab
	<i>Sorghum bicolor</i>	Burrodin	40.6 d	35.7 de
Confertiflorin		41.9 d	47.0 c	59.0 b
Desacetylconfertiflorin		49.0 cd	44.1 cd	41.4 cd
Dihydroparthenolide		89.0 a	84.0 a	78.2 a
Parthenin		48.7 cd	37.3 de	43.8 c
7-OH-3-desoxyzaluzeanin C		87.2 a	87.4 a	82.0 a
Guayulin A		70.4 b	34.4 e	33.3 de
Control		59.3 bc	59.3 b	59.3 b

^aPercent germination data are means of eight replications. Values in a column associated with a given seed and concentration and followed by the same letter(s) are not significantly different according to the Waller-Duncan Bayesian k ratio ($k = 100$) t test.

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CHOOSING APPROPRIATE METHODS AND STANDARDS FOR ASSAYING TANNIN

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Abstract—Tannins are chemically diverse polyphenolics that have multiple biological activities. Attempts to establish the ecological significance of tannins have been hindered by the complexities of tannin analysis. A multitude of analytical procedures for tannins has been described, but it is difficult for the nonspecialist to select appropriate methods. We have classified the most common procedures for determining tannin as either chemical assays, appropriate for determining the amount and the chemical nature of the tannin in a sample, or as protein-binding assays, suitable for determining the potential biological activity of the tannin in a sample. We have recommended procedures that are particularly reliable and straightforward for general use. We have also considered the problems encountered in selecting appropriate standards for tannin analysis and have recommended standards that are readily available.

Key Words—Tannin, proanthocyanidin, gallotannin, ellagitannin, protein precipitation, Folin assay, phenolic analysis.

INTRODUCTION

Investigations of the ecological or nutritional significance of tannins are often flawed by inappropriate choices of tannin assays and standards, leading to erroneous conclusions (Mole et al., 1989; Wisdom et al., 1987; Mole and Waterman, 1987a,b). A more complete understanding of the chemical structure of tannins and their chemistry will improve the quality of these studies. We present here a brief summary of the major considerations involved in choosing and interpreting methods of tannin analysis.

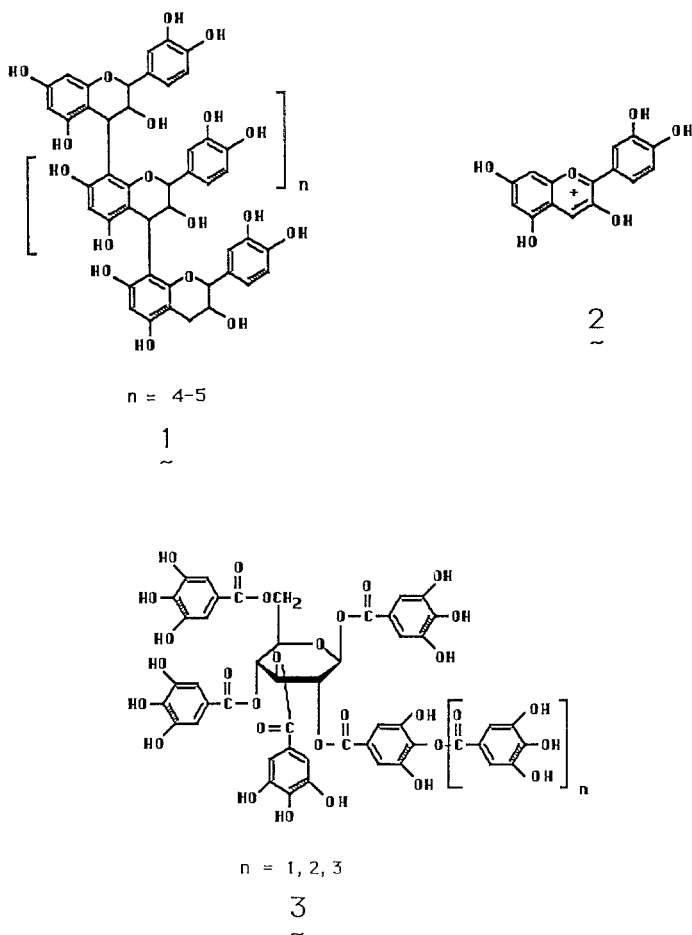


FIG. 1. Representative structures of tannins and tannin subunits: **1** is the condensed tannin (proanthocyanidin) from *Sorghum*, with polymer length of 6-7 (Butler et al., 1982); **2** is cyanidin, the anthocyanidin produced from *Sorghum* condensed tannin during the acid butanol assay; **3** is Chinese gallotannin, from one to three galloyl groups are linked as depsides (phenol-carboxylic acid esters) to the gallic acid ester on carbon 2 of the glucose in this tannin (Haslam, 1981); **4** is a simple ellagitannin, pendunculagin; **5** is gallic acid; **6** is hexahydroxydiphenic acid; and **7** is the lactone ellagic acid, which spontaneously forms from **6**.

Tannins are water-soluble polymers, rich in phenolic groups, capable of binding and/or precipitating water-soluble proteins (Bate-Smith and Swain, 1962). The two major types of tannin are chemically quite different (Figure 1). Condensed tannins (proanthocyanidins) (**1**) are flavonoid polymers, with car-

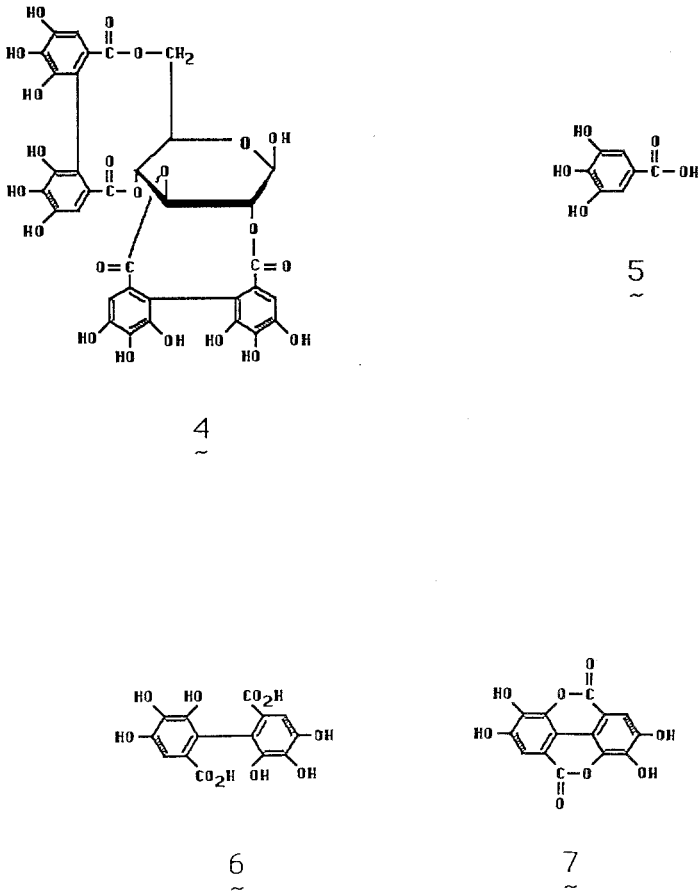


FIG. 1. Continued

bon-carbon bonds joining the individual flavonoid monomers. Condensed tannins are not susceptible to hydrolysis but can be oxidatively degraded in strong acid to yield anthocyanidins (2) (Porter et al., 1986). Hydrolyzable tannins (3, 4) are gallic or hexahydroxydiphenic acid esters of glucose or other polyols (Haslam, 1979). The ester bonds are acid, base, and enzyme labile (Haslam, 1979), and the hydrolyzable tannins are easily broken down to gallic acid (5) or hexahydroxydiphenic acid (6) subunits and the core polyol. Free hexahydroxydiphenic acid spontaneously forms the internal ester ellagic acid (7). A few examples of more complex materials, chemically related to condensed and/or hydrolyzable tannins, have been described but remain poorly characterized (Swain, 1965, 1979). Water-insoluble resins that may bind protein have been

detected (Bryant, 1981), but the structures of these materials have not been elucidated. At the present time, it is not appropriate to classify these water-insoluble materials as tannins.

Bate-Smith surveyed a large number of plants for condensed and hydrolyzable tannins and found that fungi, algae, mosses, and liverworts do not contain tannin (Haslam, 1979). More recently, tannins have been reported in some algae (Nishizawa et al., 1985). The presence of condensed or hydrolyzable tannins has been correlated with the woodiness of the plant (Swain, 1965) or with the phylogenetic rank of the plant (Haslam, 1979; Haddock et al., 1982). Results of other surveys do not suggest any taxonomic or morphological correlation with the presence of tannin (Jung et al., 1979). Conflicting conclusions in taxonomic studies of tannins are due, at least in part, to differences in assay choice and interpretation of results.

Tannins are present in the leaves, wood, flowers, or seeds of plants. Often tannins are found in only a single tissue of a plant; for example, the vegetative tissue of grasses does not contain tannin (Haslam, 1979; Watterson and Butler, 1983), but the seeds of several grasses including *Sorghum* and barley may be tannin-rich (Eggum and Christensen, 1975; Strumeyer and Malin, 1975).

Both condensed and hydrolyzable tannins interact with proteins to form soluble or insoluble complexes (Haslam, 1979). Under mild conditions, the interaction with proteins is based on noncovalent, hydrogen and hydrophobic bonds (Hagerman and Butler, 1978, 1981; McManus et al., 1981). The complexes formed can be dissociated by detergents, which disrupt hydrophobic interactions, or by high pH, which ionizes the phenolic hydroxyl and thus destroys its hydrogen-bonding ability (Hagerman and Butler, 1978, 1981). Both condensed and hydrolyzable tannins are susceptible to oxidation at high pH. The oxidation products can form covalent bonds with nucleophiles including the amino or sulfhydryl groups of proteins (Pierpoint, 1969; Leathan et al., 1980; Beart et al., 1985).

The chemical reactivity and protein-binding capacity of the tannins, their oxidation products, and, in some cases, the nontannin phenolics associated with tannins are responsible for the broad range of biological systems affected by tannins. Ecologists are interested in the role of tannins in diet selection by herbivores (Feeny, 1968; Schultz and Baldwin, 1982; Batzli, 1983; Waterman et al., 1984) and in range management (Provenza and Malechek, 1984; Owen Smith and Cooper, 1987; Robbins et al., 1987). Nutritionists need to know the effect of tannin on feed consumption, rate of weight gain, and efficiency of feed utilization by livestock (Glick and Joslyn, 1970a,b; Jambunathan and Mertz, 1973; Schaffert et al., 1974; Kumar and Singh, 1984; Mitaru et al., 1984). Agronomists investigate the effect of tannins on crop resistance to pathogens,

molds, birds, and preharvest germination (Tipton et al., 1970; Harris and Burns, 1970; Mishra et al., 1980).

In all of these fields, the major question is usually whether a particular biological characteristic is significantly correlated with the amount of tannin present. There are a variety of published methods available for analyzing tannins, and new methods appear in the literature regularly (Wisdom et al., 1987; Armory and Schubert, 1987; Makkar et al., 1987; Inoue and Hagerman, 1988). Choosing an appropriate method may seem overwhelming to the nonspecialist. None of the available methods are universally useful; for example, measurements of total phenolics correlate with biological data in some studies (e.g., McKey et al., 1978), but measurements based on protein precipitation correlate with biological value in other studies (e.g., Robbins et al., 1987). However, understanding the fundamental principles of tannin analysis will enable the investigator to select an appropriate method from a small group of well-established methods. One critical parameter to consider is whether a chemical assay or a protein-binding assay is more appropriate. Chemical assays are particularly useful for determining the amount of tannin in a sample and for elucidating the structure of the tannin. Protein-binding assays are more useful for determining the potential biological activity of tannin in a sample. The analyst must also decide whether a commercial or a noncommercial tannin standard is appropriate. Methods of tissue preservation and tannin extraction have been compared in an earlier paper (Hagerman, 1988).

Specific instructions for performing the methods discussed below, including directions for preparing the reagents and for ensuring that the methods are giving reliable data, are available in IBM-compatible form from A.E. Hagerman.

CHEMICAL ASSAYS FOR TANNIN

There are two types of chemical assays for tannin and other phenolics: General phenolic assays and specific functional group assays. These assays can be used to determine the amount of tannin and the chemical nature of the tannin in a sample. When interpreting data from chemical assays for tannin, it is essential to fully understand the specificity of each assay. Several of these assays have been reviewed (Tempel, 1982; Mole and Waterman, 1987a,b).

Redox Assays. The general phenolic assays measure properties of phenols, such as their redox potential or their ability to complex various metals. Redox assays such as the Folin (Folin and Denis, 1915; Swain and Hillis, 1959) or Prussian blue (Price and Butler, 1977) assays are used to determine total phe-

nolics. It must be recognized that these methods do not discriminate between tannin and nontannin phenolics or between phenolics and other easily oxidized material such as ascorbic acid. The two assays are similar in sensitivity; both require timed addition of several reagents, and both are susceptible to interferences from redox active compounds including ascorbic acid and thiol reagents. The nonprotein imino acid *N*-methyl-*trans*-4-hydroxyl-L-proline gives a large response with the Folin assay (Figliuolo et al., 1987), as do some common purine bases (Ikawa et al., 1988). The Folin assay was modified by Lowry (Pederson, 1979) to enhance its sensitivity to protein; both proteins and phenolics, including tannin, respond in the Lowry assay. Protein does not give a significant response in the Prussian blue assay, although purine bases do interfere (Hagerman and van Pottelsberghe, unpublished). We recommend that the Prussian blue assay be used for general phenol analysis because it is less susceptible to interference from proteins than is the Folin assay.

Metal Complexing. Assays based on the formation of colored phenolic-metal ion complexes are more specific than the redox assays since the color of the complex depends on the pattern of substitution on the phenolic ring (Wesp and Brode, 1934). Although nonphenolics such as protein do not react in these assays, nontannin phenolics respond positively. Ferric chloride reacts with phenolic compounds in alkali to form complexes with the general formula $\text{Fe}(\text{OR})_6^{3-}$, where (OR^-) represents the ionized phenol (Wesp and Brode, 1934). Various phenolics, including condensed and hydrolyzable tannins, form violet complexes with ferric ion in solutions containing sodium dodecyl sulfate and triethanolamine (Mole and Waterman, 1987a). This method is appropriate for determining total phenolics in samples containing materials that interfere with the redox methods.

In neutral solution, condensed and hydrolyzable tannins form ferric ion complexes with distinctive colors. Mole and Waterman (1987a) found that condensed tannin yields green complexes with ferric ion, while hydrolyzable tannin yields blue complexes. However, Grove and Pople (1979) point out that the color of phenolic-ferric ion complexes does not provide an unambiguous means of discriminating substitution patterns of various phenolics, so this method must be used with caution. We recommend that the more specific functional group assays described below be used to distinguish condensed from hydrolyzable tannins.

Methods for assaying and for isolating phenols based on the formation of phenol-metal ion complexes have been suggested but are not suitable for general use. A gravimetric method for total phenols based on precipitation of the phenolic by ytterbium has been described (Reed et al., 1985), but it has not been widely adopted. Although it has been suggested that lead selectively pre-

precipitates tannin, other phenols form complexes with lead (Robinson, 1980), and therefore we do not recommend that precipitation by lead be used as a criterion for distinguishing tannin from other phenols.

Functional Group Assays. The functional group assays detect and measure specific molecular structures. With functional group assays, the structural differences between condensed and hydrolyzable tannin are exploited to obtain selective methods of analysis. Although chromatographic methods have been used to detect and differentiate condensed and hydrolyzable tannins (Howard, 1987), the methods described here provide more convincing evidence for the presence of specific types of tannin.

The acid butanol assay is specific for proanthocyanidins (condensed tannins) if the optimized reaction conditions described by Porter et al. (1986) are used. This method is generally the best assay for selective determination of condensed tannins. In this assay, the flavonoid subunits of the condensed tannin polymer are oxidatively cleaved to yield the anthocyanidin; the reaction does not involve hydrolysis, and color development is decreased by water. Because color yields are dependent on the solvent, standards must be dissolved in the solvent to be used for the final analysis. Acid butanol can also be used to detect leucoanthocyanidins (flavan-3,4-diols, flavan-4-ols) by using different reaction conditions (Watterson and Butler, 1983).

The vanillin assay (Price et al., 1978) is specific for flavanols (Sakar and Howarth, 1976) and thus can be used to selectively determine condensed tannin in the presence of hydrolyzable tannin or other phenolics. Widely distributed flavanols such as catechin and epicatechin respond to this assay and could compromise the results unless an independent assay is used for confirmation. Although analogs of vanillin can be used in the assay to increase sensitivity (Putnam and Butler, 1985), the assay as described by Price et al. (1978) is adequately sensitive for most applications. The correction for "blank" color (Price et al., 1978) is essential to this method.

The vanillin and proanthocyanidin assays can be combined to provide a measure of the degree of polymerization of condensed tannin (Butler et al., 1982). This approach to determining the molecular weight of condensed tannin is feasible in situations where methods (Foo et al., 1982) relying on sophisticated spectroscopic techniques are impossible. It is particularly useful for comparing the condensed tannin from materials at different stages of plant maturity (Butler, 1982).

The recently developed rhodanine assay (Inoue and Hagerman, 1988) is specific for gallic acid esters and can be used to determine gallotannin-type hydrolyzable tannins. This assay is not subject to the ambiguities of the iodate assay previously used to determine hydrolyzable tannin (Bate-Smith, 1977; Mole

and Waterman, 1987a), and we recommend that it be adopted instead of the iodate assay. The method is not specific for tannin, since any esterified gallic acid, including low-molecular-weight, nontannin species, will react.

An appropriate assay for ellagitannin-type hydrolyzable tannins has not been devised (Bate-Smith, 1972; Mole and Waterman, 1987a). Ellagic acid can be chromatographically identified (Haddock et al., 1982).

PROTEIN-BINDING ASSAYS

Protein-binding assays can be used either to determine the amount of tannin in a sample or to determine the biological activity of the tannin. To quantitate tannin via protein binding, the amount of tannin precipitated by a standard protein is measured. For biological activity assays, either the amount of protein precipitated by the tannin in a sample is determined, or the nature of the complexes formed between tannin and protein is established. Some authors suggest use of an alkaloid, such as caffeine, instead of protein as the precipitating agent (Wall et al., 1969), but protein binding methods have been better characterized and are preferable. Several protein binding methods have been reviewed by Mole and Waterman (1987b).

Each of the protein-binding assays gives rather different responses with different preparations of tannin. The tendency of tannin to form insoluble complexes with proteins is a complex function of factors including features of the tannin (molecular weight, structure heterogeneity) and the protein (degree of glycosylation, amino acid composition, molecular weight) and the reaction conditions (pH, temperature, reaction time, relative concentrations of reactants). The complexity of the reaction between tannin and protein means that results of protein precipitation assays can be compared directly only to results obtained under virtually identical conditions. In addition, the results of those assays must be interpreted cautiously.

Tannin precipitated by protein can be measured directly in a reaction that depends on the formation of the ferric ion-phenol complex in alkaline, detergent-containing solution (Hagerman and Butler, 1978). This assay is simple to perform, gives excellent results with either condensed or hydrolyzable tannin, and is recommended as a reliable assay for most situations. However, if the procedure is modified so that a large excess of protein is used, soluble tannin-protein complexes form and complicate the data treatment (Hagerman and Robins, 1987).

The radial diffusion method (Hagerman, 1987) is a very simple assay suitable for determining insoluble tannin-protein complexes. The amount of precipitated complex is proportional to the amount of tannin in the sample. Both

condensed and hydrolyzable tannin can be determined with the method. The radial diffusion assay is particularly suitable if a large number of samples are to be analyzed or if laboratory facilities are limited.

The amount of protein precipitated by tannin-containing samples can be used to estimate the biological activity of the tannin (Robbins et al., 1987). Protein precipitated by tannin can be measured radiochemically (Hagerman and Butler, 1980a) or colorimetrically (Bate-Smith, 1973; Schultz et al., 1981; Martin and Martin, 1983; Asquith and Butler, 1985). Of these methods, the blue BSA method (Asquith and Butler, 1985) is probably the easiest to perform, but it is not very sensitive. If colorimetric methods such as the Lowry assay (Pederson, 1979) or Bradford (Coomassie blue) assay (Martin and Martin, 1983) are used to determine protein in mixtures of tannin and protein, special effort must be made to ensure that all tannin is removed from the mixture before it is assayed, since these assays respond positively to tannin as well as to protein. The radiochemical assay is the most sensitive and least susceptible to interference but requires sophisticated instrumentation and techniques.

The amount of protein precipitated is proportional to the amount of tannin present in the sample if the tannin-to-protein ratio has been set properly to control the formation of soluble tannin-protein complexes (Calderon et al., 1968; Mole and Waterman, 1987b; Hagerman and Robbins, 1987). Excess protein in the reaction mixture decreases the amount of precipitable complex that forms, resulting in an apparent decrease in the amount of tannin present. Methods like the blue BSA (Asquith and Butler, 1985) or hemoglobin precipitation assay (Schultz et al., 1981) can be used to determine the amount of tannin in a sample only under carefully controlled conditions.

The biological activity of tannin is not dependent on the formation of insoluble tannin-protein complexes. Assays that detect both soluble and insoluble complexes are therefore necessary for estimation of biological activity of tannin. Formation of soluble or insoluble tannin-protein complexes can be detected using competitive binding assays (Hagerman and Butler, 1981; Asquith and Butler, 1985), immobilized tannin (Oh et al., 1980; Austin et al., 1988), electrophoresis (Austin et al., 1988), or enzyme inhibition (Goldstein and Swain, 1965). The competitive binding assays are the most generally applicable methods for detecting soluble complexes.

The biological activity of tannin is mediated by the affinity of tannin for proteins; tannin has a high affinity for some proteins and lower affinity for other proteins. Competitive binding methods can be used to determine the affinity of protein for tannin (Hagerman and Butler, 1981; Asquith and Butler, 1985). These assays can be used to determine affinity both in systems in which soluble complexes form and in systems in which insoluble complexes form. The procedure used by Asquith and Butler (1985) is simple and recommended for most

situations. Other methods of determining binding constants include equilibrium dialysis (Barbeau and Kinsella, 1983) or ultrafiltration (Artz et al., 1987); however, it is difficult to completely eliminate possible artifacts arising from interaction between the phenolic material and the dialysis or ultrafiltration membrane. Calorimetry has been used to investigate soluble tannin-protein complexes, but it is applicable only to conditions quite unlike physiological conditions (McManus et al., 1981).

COMBINED ASSAYS

By combining chemical and protein-binding assay data, diverse tannin samples can be compared. For example, tannin-specific activity (Hagerman and Butler, 1980b) has been defined as the amount of protein precipitated by a tannin sample (Hagerman and Butler, 1980a) divided by the total phenol content of that sample (Price and Butler, 1977). That parameter can be used to compare the contamination of tannin preparations, since the tannin-specific activity increases, as nontannin phenolics are removed from the preparation (Hagerman and Butler, 1980b). The tannin-specific activity can also be used to compare preparations from different plants (Mole and Waterman, 1988).

A somewhat different parameter, the tannin-phenol ratio, has been devised to allow comparison of various tannin samples. This ratio is obtained by dividing the amount of protein precipitable phenols (Hagerman and Butler, 1978) by total phenols (Price and Butler, 1977). This ratio has been used (Butler, 1982) to establish the amount of nontannin phenolics in crude plant extracts, but was incorrectly called tannin-specific activity in that paper.

For condensed tannins, the precipitation-size ratio, or amount of protein precipitated (Hagerman and Butler, 1980a) divided by the flavan-3-ol end units (Butler et al., 1982), can be used to determine how activity changes as the molecular weight of the tannin changes (Table 1). It is generally believed that moderate-sized condensed tannin polymers precipitate protein more efficiently than either very small or very large polymers (Haslam, 1979).

TANNIN STANDARDS

It is a practical impossibility to devise a simple extraction procedure that yields chemically pure tannin, so the analyst must devise a way to determine tannin in crude extracts that contain tannin, tannin-complexed proteins, and nontannin phenolics. The crude extracts must be compared with standard tannins in order to make estimates of the tannin content of the samples, expressed in terms of the chosen standard. Selection of a suitable standard is critical if meaningful

TABLE 1. PROTEIN PRECIPITATED PER FLAVANOL END GROUP^a

Tannin	Chain length	BSA ppt/ flavanol end group ^b (mg/A520)
Pinto bean	2.9	257
Sorghum	4.2	672
Quebracho	5.1	733
Wattle	5.9	553

^aCalculated from Asquith and Butler (1985).

^bFlavanol end groups measured as absorbance at 520 nm with the vanillin assay in HOAc.

results are to be obtained from the analyses. Two types of tannin standards can be used: absolute standards and relative standards.

Suitable tannin standards for determining the *absolute* amount of tannin in a given plant could be obtained only by purifying the standard from the plant of interest, a procedure recommended by Wisdom et al. (1987). However, that approach is not simple. Most plants contain several chemically distinct types of tannin, often including both condensed and hydrolyzable tannins (Haslam, 1979). Not only may the ratio of the chemical species change with environmental, physiological, and seasonal changes, but also the extractability of the various molecular forms may change (Hatano et al., 1986; Baldwin et al., 1987; Hagerman, 1988). Adequate characterization of the pure tannin obtained is necessary to ensure that it is truly pure and to identify all the chemical species present. Even in simple systems containing only condensed tannin (e.g., *Sorghum* grain) obtaining pure tannin is laborious (Hagerman and Butler, 1980b). For more complex plant systems, obtaining a representative pure tannin standard for the absolute determination of tannin may well be impossible.

Using commercially available standards, relative amounts of tannin can easily be determined. The assay is standardized with the commercial standard, and the tannin in the plant extracts is then reported in terms of the weight of the selected standard. Nontannin phenolics are sometimes adequate standards. For example, the rhodanine assay and the various total phenol assays can be standardized with reagent-grade gallic acid, and the vanillin assay can be standardized with commercial catechin. The acid butanol assay can be standardized with commercial cyanidin, although that standardization would be most appropriate for procyanidin, not for prodelphinidins or other proanthocyanidins.

Tannic acid is probably the best commercially available tannin standard,

but ideally the commercial material should be purified. A simple purification using Sephadex LH20 (Hagerman and Klucher, 1986) yields an appropriate standard for the rhodanine assay, for any of the redox assays, or for any precipitation assay. Tannic acid cannot be used to calibrate the acid butanol assay. Unpurified commercial preparations of tannic acid contain variable amounts of low-molecular-weight galloyl esters and of nontannin material (Table 2). Each of these preparations is a unique mixture of galloyl esters, and each has a different ability to precipitate protein (Weerasuriya, Wilson and Hagerman, unpublished). If unpurified commercial tannic acid is used as a standard, its source must be indicated clearly because the preparations are so variable in composition.

Although tannic acid is an appropriate standard for analysis of tannins, it may be an inappropriate material to use for *in vivo* tests of the biological activity of tannin. Recent work (Robbins and Hagerman, unpublished) suggests that tannic acid does not affect nitrogen utilization in ruminants, whereas other hydrolyzable tannins diminish nitrogen utilization. It is not surprising that a material might be appropriate as a chemical standard but quite inappropriate for bioassay.

Sorghum tannin is an appropriate standard condensed tannin, but it is not available commercially and is rather tedious to purify (Hagerman and Butler, 1980b). Quebracho tannin is the only readily available condensed tannin. It must be purified (Asquith and Butler, 1985) because it contains large amounts of nontannin materials. There are probably substantial differences among the materials provided by various suppliers and even among various lots from a single supplier. Since a single commercial standard is not available, we suggest that all workers start to use purified quebracho standard prepared from a single

TABLE 2. GALLIC ACID IN VARIOUS PREPARATIONS OF TANNIC ACID^a

Tannic acid source	Total GA (mg/mg TA)	Ester GA (mg/mg TA)
Sigma (pfs) ^b	0.633	0.546
MCB reagent	0.689	0.598
Mallinckrodt technical ^c	0.691	0.610
Mallinckrodt reagent	0.449	0.413
Mallinckrodt purified ^d	0.809	0.766

^aTotal gallic acid and free (unesterified) gallic acid were determined with the rhodanine assay (Inoue and Hagerman, 1988) in triplicate for each sample of tannic acid, and esterified gallic acid was calculated by difference. TA = tannic acid, GA = gallic acid.

^bFrom Ken Albrecht, University of Wisconsin.

^cFrom Charles Robbins, Washington State University.

^dBy the method of Hagerman and Klucher (1986) (fraction 3).

lot of the crude commercial mixture. A.E. Hagerman will provide the crude material and instructions (Asquith and Butler, 1985) for preparation of the purified standard to any interested laboratories. Use of a single standard should significantly reduce confusion about analysis of condensed tannin.

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REDUCTION IN DIET BREADTH RESULTS IN
SEQUESTRATION OF PLANT CHEMICALS AND
INCREASES EFFICACY OF CHEMICAL DEFENSE IN
A GENERALIST GRASSHOPPER

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Abstract—The lubber grasshopper, *Romalea guttata*, is a generalist feeding on a broad diet of many herbaceous plant species and has a metathoracic defensive secretion normally containing phenolics and quinones synthesized by the insect. When insects were reared on a restricted diet of wild onion, they sequestered sulfur volatiles from the plant into their defensive secretions. These compounds were not detected by gas chromatography–mass spectroscopy in secretions of insects on an artificial diet or a natural, generalist diet of 26 plants that included wild onion as a component, nor were they present in secretions from field-collected insects. Defensive secretions of insects reared on wild onion were significantly more deterrent, by as much as an order of magnitude, to two species of ant predators than secretions from insects on either of the other two diets, despite a reduction in the concentration of autogenous defensive chemicals in secretions of insects on the onion

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diet. Sequestration of plant chemicals that increased defensive efficacy occurred when diet breadth was reduced. We suggest that this occurs because under conditions of specialization, plant secondary metabolites are more likely to be ingested and bioaccumulated in sufficient concentrations to have biological activity against predators. What we define as casual bioaccumulation of bioactive plant chemicals following dietary specialization may lead to evolution of sequestered defense syndromes in insects, and this process may not necessarily require specific adaptation to or coevolution with a toxic host plant.

Key Words—Lubber grasshopper, *Romalea guttata* (microptera), Orthoptera, Acrididae, onion, generalist diet, artificial diet, sulfur volatiles, ant predation, casual bioaccumulation, specialization, evolution of defenses.

INTRODUCTION

Many herbivorous insect species use chemical defenses against predators (Duffey, 1976; Blum, 1981; Pasteels et al., 1983a). In some cases, autogenous defensive chemicals are made by the insect from dietary precursors. Alternatively, chemicals from the diet may be sequestered into the body tissues, fluids or glands with or without modification by the insect. Examples of sequestered defenses include cardenolides in the monarch butterfly (Brower, 1984), milkweed bug (Duffey and Scudder, 1972), and pyrogomorphid grasshoppers (Rothschild and Parsons, 1962); salicylaldehyde in some chrysomelid beetles (Pasteels et al., 1983b); and cardenolides and pyrrolizidine alkaloids in some arctiid moths (Rothschild and Aplin, 1971).

The ecological factors that initiate or constrain evolution of the sequestered defense syndrome are of considerable interest but poorly understood. It has been suggested that sequestered defenses initially evolved as a special insect adaptation for avoiding toxicity of specific chemicals in the host plant, by placing these chemicals out of harm's way in tissues or fluids. Storage of these compounds then subsequently conferred a selective advantage against predators (Brower and Brower, 1964; Rothschild, 1973). An alternative possibility is that the sequestered defense syndrome may arise as a consequence of what we term initial casual sequestration or bioaccumulation of ingested chemicals in body tissues (Duffey, 1976, 1980). The chemicals may not necessarily be toxic to the insect, and specific adaptation to a host plant may not be necessary. Bioaccumulated compounds might then deter predators if they were sequestered in sufficient concentrations. In this article we present evidence that two key characteristics of a sequestered defense syndrome—incorporation of plant chemicals into defensive secretions, and a concomitant increase in defensive efficacy against predators—can arise in an insect when there is no evidence for specific adaptation to a plant species, when the plants are not toxic to the insect, and in

a manner best described as casual sequestration. Furthermore, we show that diet breadth is the major ecological variable that determines whether or not sequestration of at least one class of plant chemicals occurs in sufficient amounts to have effects on predators.

The lubber grasshopper, *Romalea guttata* (Houttuyn) [Orthoptera; Romaleidae = *R. microptera* (Beauvois)] (Kevan, 1980), is a large, flightless, generalist grasshopper with a local host range of over 80 species of herbaceous and woody plants. Many of these plants will be eaten within the lifetime of an individual insect (C.G. Jones, D.W. Whitman, M.S. Blum, unpublished data). Adult *Romalea* have an impressive defensive armory, including warning coloration, a pink wing-flashing display and sharp spines on the legs. Most notable is the odoriferous, frothy, glandular secretion produced with an audible hiss from modified metathoracic spiracles (Eisner et al., 1971). This secretion is deterrent to ants and other predators (Eisner et al., 1971; D.W. Whitman, C.G. Jones, M.S. Blum, unpublished data). The major components of the secretion of wild insects are various phenolics and quinones, including hydroquinone, catechol, *p*-benzoquinone, phenol, guaiacol, and 4-methoxybenzaldehyde (Eisner et al., 1971; Jones et al., 1986, 1987). These compounds are presumably autogenous because they are found in the secretions of insects reared on an artificial diet that does not contain any of these chemicals (Jones et al., 1987). The autogenous defensive components are markedly affected by diet breadth. Restricting *Romalea* to a single host plant or artificial diet results in a reduction in the number and concentration of compounds, and thus alters the composition of the secretion, compared to lubbers reared on a generalist diet or collected in the field (Jones et al., 1987).

Eisner et al. (1971) showed that *Romalea* collected from an area sprayed with the herbicide 2,4-D had 2,5-dichlorophenol in the defensive secretion. We therefore wondered if *Romalea* was capable of sequestering natural plant products into the defensive secretion. In our studies on the effects of diet breadth on autogenous defenses (Jones et al., 1987), we noticed that the secretions of insects reared on a single host plant diet of onion appeared to have an onionlike odor. Here, we evaluated the possibility that sequestration of onion compounds might have occurred. We reared *Romalea* from first instar to adult on three different diets: (1) a restricted single plant species diet of wild onion, *Allium canadense* L., that we have observed is one of a number of favored food plants of *Romalea* (C.G. Jones, D.W. Whitman, M.S. Blum, unpublished data); (2) a "natural" generalist diet of plant species used by *R. guttata* in the field, which included wild onion as a component species; and (3) a restricted artificial diet known to lack any of the insects' autogenous phenolics and quinones and lacking any plant secondary metabolites. We compared the chemistry of secretions from insects reared on the different diets. We then bioassayed the secretions against a known ant predator of *Romalea*, the red imported fire ant, *Solenopsis invicta*

Buren (Formicidae: Myrmicinae), and a less aggressive ant species, *Tapinoma melanocephalum* F. (Formicidae: Dolichoderinae). Our findings are relevant to the ecology and evolution of chemical defense in insects.

METHODS AND MATERIALS

Rearing of Insects. Lubber grasshoppers were reared under the same conditions as used by Jones et al. (1987). The first of the three diets consisted of freshly cut leaves of *Allium canadense* in water. The second diet consisted of 26 species from 15 families of shrubs, herbs, forbs and grasses known to be eaten by *Romalea* in the field (C.G. Jones, D.W. Whitman, M.S. Blum, unpublished data). Plants were collected daily and the petioles or stems placed in water. The species list was as in Jones et al. (1987) and included wild onion, *Allium canadense*. The third diet was an artificial diet (Dadd, 1960) containing cellulose, sucrose, dextrin, mineral salts, cholesterol, linoleic acid, casein, pectone, egg albumin, ascorbic acid, and B vitamins and was fed as a water-moistened cake. Ten males and 10 females were also collected from the field for chemical analysis of their secretions.

Secretion Collection. Secretions were collected from adults at the eighth day after the terminal molt, by gently squeezing the thorax while applying a 10- μ l microcapillary to the metathoracic spiracule orifices. A single milking removes all the secretion produced since the last molt. Molting insects discard all previous secretions. The volume collected (range 1–8 μ l) was recorded and the secretion sealed in the microcapillary and stored at -10°C . Secretions from insects reared in the same batch on the different diets were randomly allocated for: (1) separately pooled secretions of males and females for mass-spectral analysis, (2) secretions from individual females and pooled secretions of females for quantitative analysis of sequestered compounds, (3) secretions from individual females for quantitative analysis of autogenous compounds, and (4) pooled secretions of females for bioassays. In addition, separately pooled secretions of wild male and female insects were subjected to mass-spectral analysis.

Qualitative Analysis of Secretions. Compounds were identified from splitless injection (10 psi) of undiluted secretion on a Finnigan 4021 gas chromatograph-mass spectrometer (GC-MS) using a 30-m \times 0.3-mm-ID DB-5 fused silica bonded phase column (J&W), programmed at 50°C for 1.5 min, then $100\text{--}250^{\circ}\text{C}$ at $15^{\circ}\text{C}/\text{min}$. The GC-MS was operated in electron impact mode (70 eV, 1400 V, 10^{-7} amp/V; scanning 45–350 amu, 1.5 sec/scan) with acquisition commencing at sample injection. Compounds were identified by using the molecular ions and characteristic mass fragments, by comparison with library spectra (*r* fit), and by GC-MS of authentic standards. Pooled secretions col-

lected from 10 individual males and females reared on each of the three diets and 10 males and females collected in the field were analyzed by GC-MS.

Quantitative Analysis of Sequestered Compounds. Diethyl ether extracts of secretions were analyzed by subambient, capillary gas chromatography (GC) (Varian 6000), using a 30-m \times 0.32-mm-ID fused silica bonded phase column (J&W, DB-5), helium carrier (2 ml/min) splitless injection (200°C, purge at 0.7 min), and flame ionization detection (300°C, 1×10^{12} sensitivity). Initial column temperature was 20°C, ramped to 40°C at 10°C/min, then 40°C/min to 200°C and held at 200°C for 2 min. Secretions from eight individual females and the pooled secretions of three females reared on the onion diet were each extracted with 10 μ l redistilled diethyl ether and immediately analyzed by GC. The 2.5- μ l aliquots were injected, and exact volumes of ether recovery and reproducibility were determined by addition of *dl*-alpha pinene to the ether as an internal standard, prior to extraction (325 μ mol/liter). We determined the concentration of eight sulfur-containing volatiles as the mean of three replicate injections: 1-propanethiol (minimum detectable quantity per microliter, 14 pg), methylthiirane (5 pg), methyldisulfide (21 pg), isopropylsulfide (11 pg), 2,5-dimethylthiophene (30 pg), methylpropylsulfide (13 pg), isopropylsulfide (28 pg), propylsulfide (25 pg). Retention times were accurate to $\pm 1\%$ ($P = 0.05$); peak heights with tangent baseline correction were accurate to $\pm 10\%$ ($P = 0.05$), based on analysis of standards and replicate injections of pooled samples, and were measured on a Varian 402 integrator. Retention times and concentrations of compounds were determined by calibration against authentic standards and confirmed by using coinjection and independent injection. Standards were commercially available, except for methylpropylsulfide, which was synthesized by the method of Carson and Wong (1959). Combined concentrations were determined from the sum of the concentrations of each compound.

Quantitative Analysis of Autogenous Compounds. Secretions were analyzed for six phenolics and quinones (hydroquinone, *p*-benzoquinone, phenol, catechol, guaicol, 4-methoxybenzaldehyde) using tertiary gradient reverse-phase high-performance liquid chromatography (HPLC) on a C_{18} bonded-phase silica column with a water-methanol-1% H_3PO_4 convex elution gradient, according to the method of Jones et al. (1986, 1987). Secretions from 10 individual females reared on each diet were diluted to 1% in 18 mohm deionized water, and triplicate 10- μ l aliquots were analyzed. Combined concentrations of phenolics and quinones were determined from the sum of the concentrations of each compound.

Bioassay of Secretions. In preliminary binary choice experiments, we determined that ants preferred pure sucrose solutions to those containing wild *Romalea* defensive secretions (D.W. Whitman, C.G. Jones, M.S. Blum,

unpublished data). We therefore decided that relative deterrence of the three different secretions was best determined by simultaneously comparing the secretions against one another, when they were made up in sucrose solution. Five laboratory colonies of *Solenopsis invicta*, varying in size from 1000 to 4000 individuals, were each presented with a 45 × 75-mm glass slide containing three separate 50- μ l droplets of 4% sucrose. Each separate droplet contained 1 μ l of *Romalea* metathoracic secretion pooled from individual females reared on either the natural, the onion, or the artificial diet. Ants foraged down a common trail to this comparative bait station. The common foraging trail prevented recruitment to one bait from affecting the total number of foragers approaching the bait station and reduced any biases due to the deterrence of a given bait. The number of ants feeding on each droplet was recorded every 4.5 min, up to the time that the first of the sucrose baits with secretion was completely consumed. Trials were repeated three times for each colony, with the order of the three droplets on the slide being changed for each trial. It was determined that the number of ants visiting the station initially increased, stabilized by 10 min, and remained more-or-less constant for over 30 min. Therefore the number of ants at each bait at 40 min was recorded for each colony and trial. These data were analyzed using ANOVA followed by multiple comparisons (least-significant-difference tests).

Two laboratory colonies of *Tapinoma melanocephalum* (colony size no more than 3000 individuals) were tested using the above procedures. Ten percent sucrose solution containing secretions was used because this species of ant did not recruit sufficient numbers to 2% pure sucrose solution to permit adequate evaluation of deterrence. Trials were repeated twice. In addition, pairwise comparisons of artificial diet secretion (1 μ l) + 10% sucrose versus natural diet secretion + sucrose, and natural diet + sucrose versus onion diet + sucrose were also carried out using the glass slide technique. Three lab colonies were used with five repeated trials each. Again, the number of ants visiting the baits at 20 min was recorded. Data in these trials were analyzed as above.

RESULTS

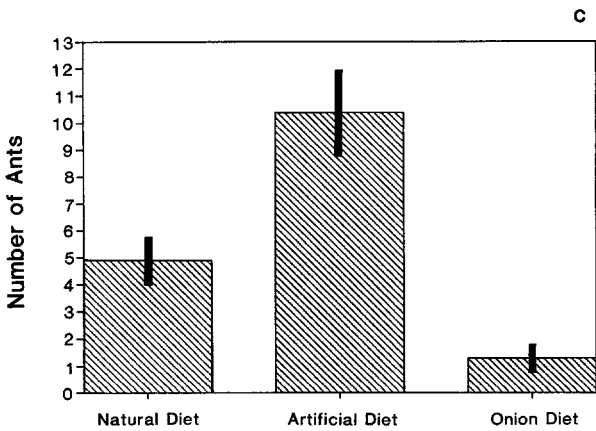
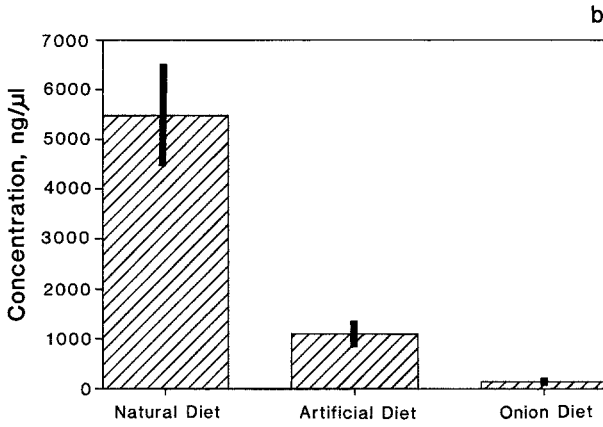
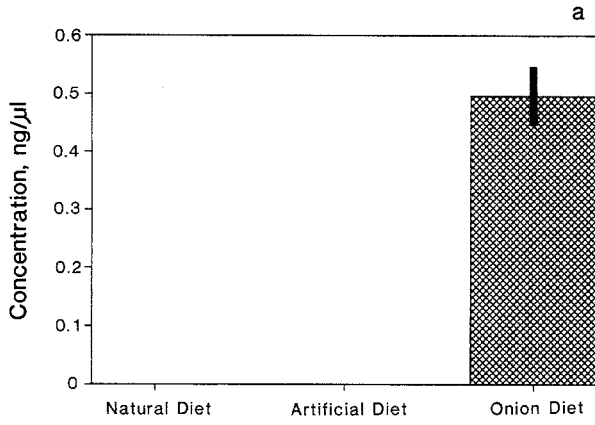
Sequestration of Plant Compounds. The secretions of insects reared on the wild onion diet lacked the characteristic color and odor of secretions from insects on the natural diet and had a marked onionlike odor. GC-MS of the volatile fraction of pooled secretions from males and females showed the presence of a large number of sulfur-containing compounds (C.G. Jones and P.J. Silk, unpublished data). Eight compounds were unambiguously identified (isopropylsulfide, methylpropylsulfide, isopropylsulfide, methylsulfide, propylsulfide, propanethiol, methylthiirane, and 2,5-dimethylthiophene) and were

identical to those previously isolated and identified from onion (Carson and Wong, 1961; Brodnitz et al., 1969; Boelens et al., 1971). The total concentrations of these eight onion sulfur volatiles that were generally the most abundant in the secretions were quantified in the defensive secretions of eight adult female individuals reared on the onion diet. Mean concentration of all eight of these compounds combined was 0.5 ng/ μ l (Figure 1a). None of these sulfur volatiles, nor any other sulfur-containing compounds, were detected in GC-MS analyses of pooled secretions from male or female insects reared on the natural or artificial diet (pooled from 10 of each sex on each diet), or from secretions pooled from 10 individuals of each sex of field-collected insects occurring in an area where a diversity of plant species grow, including wild onion. In a separate rearing experiment, sulfur volatiles were detectable by odor and GC-MS in defensive secretions of insects within five days of transfer from artificial to onion diets.

Autogenous Compounds. Secretions from insects raised on either onion or the artificial diets contained significantly lower concentrations of all autogenous phenolics and quinones than secretions from insects on the generalist, natural diet. Combined phenolic and quinone concentrations were depressed fivefold on the artificial diet and 37-fold on the onion diet (Figure 1b). The concentrations of autogenous compounds in secretions of insects on onion diets were also significantly lower than concentrations in artificial diet secretions (t tests, $P < 0.0001$). These new data agree with our previous findings for autogenous compounds (Jones et al., 1987).

Efficacy of Defensive Secretions. To determine whether changes in the secretion chemistry affected biological activity, we bioassayed secretions against two ant species. The imported fire ant, *Solenopsis invicta* Buren, is a very aggressive predator of insects (Lofgren et al., 1975). This ant species did not evolve in the same habitat as *Romalea guttata*, but we have observed predation by *Solenopsis* on *Romalea* in the field (D.W. Whitman, C.G. Jones, M.S. Blum, unpublished data). *Tapinoma melanocephalum* F. is a less aggressive, omnivorous scavenger sympatric with *Romalea* in Florida (Creighton, 1950). We estimated the relative deterrence of the defensive secretions. With *Solenopsis invicta*, significantly more ants visited and fed at the artificial diet secretion bait compared to the natural diet secretion bait, and significantly more ants visited and consumed both of these baits compared to the onion diet secretion bait (Figure 1c shows means \pm 1 SE). Overall effect of treatments was significant: ANOVA: $F = 16.42$, $df = 44$, $P = 0.0001$. All means in multiple comparisons were significantly different from each other, LSD t tests, $\alpha = 0.05$.) Approximately 63, 30, and 7% of all foraging ants visited and fed at the artificial, natural, and onion diet secretion baits, respectively.

The less aggressive ant, *Tapinoma*, showed even greater differences between treatments. In trials where all three secretion baits were presented



together, the proportion of ants visiting and feeding at the baits was approximately 70 (artificial diet), 29 (natural diet), and 1% (onion diet) (mean \pm 1 SE number of ants at bait at 20 min for two colonies and two trials was: artificial 13.3 ± 3.4 ; natural 5.0 ± 1.6 ; onion 0. ANOVA: $F = 9.55$, $df = 11$, $p = 0.006$. All pairs of means in multiple comparisons were significantly different from one another, LSD t tests, $\alpha = 0.05$). In comparisons with *Tapinoma*, where pairs of secretions were tested together, the number of ants at artificial diet secretion baits at 40 min (mean \pm 1 SE = 14.9 ± 1.7) was significantly greater than the number at natural diet secretion baits (mean \pm 1 SE = 7.0 ± 1.4 ; ANOVA: $F = 8.43$, $df = 49$, $p = 0.007$). Numbers at 40 min at natural diet secretion baits (mean \pm 1 SE = 45.1 ± 4.4) were significantly greater than numbers at onion diet secretion baits (mean \pm 1 SE = 0.3 ± 0.16 ; ANOVA: $F = 35.49$, $df = 49$, $p = 0.0001$).

DISCUSSION

Diet clearly affects the quality and quantity of the chemical components of the defensive secretion of *Romalea* and the efficacy of the defensive secretion against these predators. Sequestration of measurable quantities of sulfur compounds occurred only when *Romalea* specialized on the onion diet. The natural diet also included wild onion as a component species, and yet secretions from insects reared on this diet did not contain any detectable sulfur-containing compounds, nor were these compounds found in field-collected insects that fed on a large number of plant species. Since these compounds were not present in the artificial diet, their absence from secretions of insects specializing on this diet was to be expected. In addition, specialization on either artificial or onion diets again caused a reduction in the concentrations of autogenous phenolics and quinones, as has been previously reported (Jones et al., 1987).

The response of ants to baits containing these secretions is consistent with the chemical differences resulting from the presence of sequestered compounds and the depression of autogenous compounds. Sequestration of sulfur com-

FIG. 1. (a) Mean combined concentrations of eight sulfur-containing volatiles (ng/ μ l secretion, \pm 1 SE) in defensive secretions of individual adult female *R. guttata* ($N = 8$), reared on an onion diet. Pooled secretions of females ($N = 10$) on the other diets did not contain detectable levels of sulfur volatiles. (b) Mean combined concentrations of six autogenous phenolics and quinones (ng/ μ l secretion, \pm 1 SE) in defensive secretions of adult female *R. guttata* ($N = 10$), reared on three diets. (c) Mean number of *Solenopsis invicta* ants (\pm 1 SE, five colonies, three trials each) visiting and feeding at 20 min on sucrose baits containing defensive secretions of *R. guttata* females reared on three diets.

pounds in onion diet secretions is associated with an increase in defensive efficacy compared to natural diet secretions, despite depression of the concentrations of autogenous phenolics and quinones. Reduction of autogenous phenolic and quinone concentrations in artificial diet secretions is associated with a reduction in the efficacy of defense compared to natural diet secretions. The deterrent activity of components sequestered on the restricted onion diet appears to be much greater than the activity of the autogenous compounds. The combined sequestered sulfur compounds occurred at concentrations about 10^4 lower in onion diet secretions than combined concentrations of phenolics and quinones in the natural diet, and yet the onion diet secretion bait was visited and fed on by *Solenopsis* at about one quarter the rate of the natural diet secretion bait.

Dietary specialization in *R. guttata* therefore led to rapid and simultaneous changes in two attributes important to its chemical defense: acquisition of compounds directly from the diet and a reduction in autogenous compounds. This led to a concomitant increase in defensive efficacy. Experimental specialization in *Romalea* produces results strikingly similar to many specialist insect herbivores where chemical defense against predators depends upon sequestration of host-plant chemicals (Brower, 1984; Rothschild, 1973; Roeske et al., 1976; Duffey, 1976, 1980; Blum, 1981).

Why should specialization result in sequestration in *Romalea*? We propose that the degree of sequestration of plant chemicals in this insect is primarily a function of the concentration of compounds ingested in the diet. When a generalist diet is consumed, compounds from an individual plant species may be too dilute to be detectable or biologically active against predators in comparison to the autogenous compounds. Conversely, specialization results in the ingestion of sufficiently high concentrations of compounds from one plant species such that these compounds may be sequestered in detectable levels that have biological activity. Interestingly, data from Eisner and coworkers (1971) may be relevant to our contention. They reported 2,5-dichlorophenol in the defensive secretion of *R. guttata* collected in herbicide-sprayed areas and suggested that this compound was derived from the herbicide 2,4-D. 2,5-Dichlorophenol was not present in secretions of insects from unsprayed areas. Although in this case *Romalea* was consuming a generalist diet, it may have been, in effect, specializing on a halogenated phenol diet because all of the plants were sprayed with herbicide.

Our results may have important implications for understanding the evolution of plant-derived chemical defense in insects. What we term casual bioaccumulation of plant chemicals active against predators may be an important process that initiates selection toward the plant chemical-based defenses found in many insects. Bioaccumulation of natural and synthetic chemicals is a widespread phenomenon (Duffey, 1980). A large number of plants contain high levels of biologically active compounds, and a general relationship between

concentration of such compounds in the diet and subsequently in the body does exist for many organisms (Duffey, 1980). It follows that situations in which a high concentration of compounds are ingested may be more likely to lead to concentrations of plant compounds in herbivorous insects that are active against predators. Our data on *R. guttata* suggest that the ecological equivalent of this dose relationship is diet breadth. Generalist consumers may be less likely to sequester chemicals in bioactive concentrations than their specialist counterparts because the generalist diet dilutes the concentrations of specific compounds ingested from each plant. It is possible that initiation of the evolution of sequestered defenses may arise when an insect feeds on a single host plant or limited numbers of species because casual sequestration of chemicals occurs in sufficient amounts for these compounds to be active against predators. If so, evolution of sequestered defenses may not necessarily initially require a high degree of adaptation or coevolution with a toxic host plant, as has been previously suggested.

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JUGLONE REDUCES GROWTH, NITROGENASE ACTIVITY, AND ROOT RESPIRATION OF ACTINORHIZAL BLACK ALDER SEEDLINGS

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Abstract—European black alder trees [*Alnus glutinosa* (L.) Gaertn.] fix nitrogen with nodular symbionts and are interplanted with valuable black walnut trees (*Juglans nigra* L.) to increase soil nitrogen fertility. However, on some soils interplanted alder can be killed by black walnut's allelochemical juglone. In order to better understand the effects of juglone directly on the growth, nitrogen fixation, and root respiration of black alder, we grew nodulated alders hydroponically in a nitrogen-free nutrient solution at juglone levels of 2×10^{-6} , 2×10^{-5} , and 0 molar (M). Results indicate that nitrogenase activity (acetylene reduction) of alders growing in 2×10^{-5} M juglone was reduced relative to alders without added juglone after one day, and in 2×10^{-6} M juglone after five days. Root respiration (CO_2 evolution) and the relative increase of plant fresh weight were reduced in the 2×10^{-5} M juglone treatment. In a related experiment, black alder germinants were grown in Flanagan silt loam soil dosed with 10^{-3} , 10^{-4} , and 0 M juglone. The inhibitory effects of 10^{-3} M juglone on radicle elongation ceased 22–37 days after juglone treatments were started, suggesting that this soil can readily detoxify juglone.

Key Words—Acetylene reduction, allelopathy, *Alnus glutinosa*, black walnut, *Frankia*, *Juglans nigra*, juglone, nitrogen fixation, root respiration.

INTRODUCTION

Black walnut (*Juglans nigra* L.) is able to inhibit the growth of many plant species (Cook, 1921; Strong, 1944; Gabriel, 1975) through its allelochemical juglone (5-hydroxy-1,4-naphthoquinone). In living tissue this compound is in

a reduced, nontoxic form called hydrojuglone (Gries, 1943; Lee and Campbell, 1969), but when plant tissue is incorporated into the soil, hydrojuglone is oxidized and toxic juglone is released (Rietveld, 1983).

Plant species show varying responses to the presence of juglone (Funk et al., 1979; Rietveld, 1983). The effect of juglone on black alder trees [*Alnus glutinosa* L. (Gaertn.)] is of particular interest because of its use as a nitrogen-fixing intercrop with commercially valuable black walnut. Of 16 herbaceous and woody species tested for juglone sensitivity, black alder was one of five most inhibited in seedling shoot elongation and dry weight accumulation, showing depressed growth at a concentration as low as 10^{-6} M juglone (Rietveld, 1983). Rietveld et al. (1983) also reported the sudden decline and death of black alder as a result of juglone toxicity in four mixed plantings with black walnut 8–13 years after planting.

Juglone also affects the nitrogen-fixing endophyte of black alder. A concentration of 10^{-4} M juglone inhibited in vitro growth of the symbiotic actinomycete *Frankia* (Dawson et al., 1981; Dawson and Seymour, 1983; Vogel and Dawson, 1985, 1986). Nodulation of black alder by *Frankia* was also significantly depressed by the addition of 10^{-4} M juglone to soil (Vogel and Dawson, 1985).

One mechanism by which juglone is thought to affect plant growth is through a reduction in respiration. Perry (1967) reported the inhibition of oxygen uptake by leaf disks of tomato (*Lycopersicon esculentum* Mill.) and bean (*Phaseolus vulgaris* L.) at juglone concentrations greater than 10^{-5} M. Similarly, juglone concentrations as low as 5×10^{-5} M reduced O_2 uptake in excised corn roots (Köeppe, 1972).

Edaphic conditions may influence the toxicity of juglone. On well-drained soils, red and white pine (*Pinus resinosa* Ait. and *P. strobus* L.) did not exhibit any adverse allelopathic effects when interplanted with walnut, but on imperfectly and poorly drained soils, pines growing with walnut showed poorer survival and growth than pines without adjacent walnut (Fisher, 1978). In the same study, a bioassay of "wet" and "dry" soils dosed with juglone showed that inhibition of radicle extension in red pine and detection of juglone (using thin-layer chromatography) occurred for a substantially longer period of time in the "wet" soil. Fisher considered this evidence that microbial degradation of juglone is determined by the moisture regime and, hence, aeration of the soil. He thought it less likely that differences in soil moisture had affected the sorption of juglone by the soil. Microorganisms that break down aromatic and phenolic substances similar to juglone are aerobic heterotrophs (M. Alexander, 1977). Schmidt (1988) isolated a bacterium (*Pseudomonas* J1) from soil beneath black walnut trees that could grow rapidly using juglone as its sole source of carbon and energy.

As well as possibly limiting microbial degradation of juglone, a wet soil will reduce the O_2 available for root respiration. What effect the presence of

juglone may have on root respiration of trees, in particular trees growing under reduced-oxygen conditions as occur in wet soils, is unknown. But given that O_2 uptake of other plants and plant tissues is reduced in the presence of juglone, it is likely that reductions in root respiration will occur in soil or other substrates as oxygen levels decrease. In nature, low oxygen conditions in wet soils may both slow juglone degradation and compound the inhibitory effects of juglone on plant respiration.

We hypothesized that the toxicity of juglone to black alder germinants would decline over time after dosing a fertile soil suitable for walnut culture and that the rate of that decline would be lower in wet soil. We also hypothesized that growth, acetylene reduction, and root respiration of nitrogen-fixing black alder grown hydroponically would be reduced when juglone is added and when the level of oxygen available to the roots is reduced.

The first experiment was designed to assess the rate at which a fertile soil detoxifies added juglone. The second experiment in hydroponics excluded the effects of microbial degradation and soil sorption of juglone and examined the direct effects of juglone and oxygen level on nitrogenase activity in alder root nodules and respiration in alder roots.

METHODS AND MATERIALS

Experiment 1. An experiment to measure the changes in juglone toxicity over time in wet and dry soils was carried out in a temperature-controlled environment, based on a completely randomized design with two juglone levels and a control, two soil moisture regimes, five replications, and four time intervals.

Soil for the experiment was a Flanagan silt loam (Aquic Argiudoll), somewhat poorly drained, with an average surface organic matter content of 4.5% and high available water capacity (J.D. Alexander et al., 1974). It was taken from a site close to Urbana, Illinois, and was collected using a soil coring device 1.6-cm in diameter. The cylindrical soil cores of length 10 cm and volume 80.5 cm³ were placed over 2 cm³ of glass beads inside test tubes of the same internal diameter and incubated at 27°C. Air dry weights of soil cores ranged from 20 to 26 g.

The three soil treatments consisted of a control of distilled water without juglone, 10^{-4} M and 10^{-3} M juglone. A 10^{-3} M solution is at the limit of juglone solubility in water. After determining the field capacity of the soil, soil cores were allowed to dry to 30% of their field capacity, after which 6 ml of the appropriate juglone or control solution were added. This brought the moisture in the soil up to field capacity.

Two soil moisture treatments were used. In the "wet" regime, there was a cycle in which the soil was dried to 90% of field capacity and was rewet with distilled water to field capacity. In the "dry" regime, soil was dried to 50% of field capacity and was rewet with distilled water to 70% of field capacity. Loose-

fitting caps were used for both treatments, allowing gas exchange while maintaining a high relative humidity inside the test tubes.

At intervals of 4, 22, 37, and 52 days after treatment, a bioassay of the soil was performed using black alder seedlings three days after germination with radicles about 1 mm in diameter. Soil was removed from the test tubes and placed in Petri dishes 5 cm in diameter. After measuring their initial radicle lengths, seven black alder germinants were sown in each dish, and the dry soil was brought up to field capacity. Dishes were kept in a growth chamber at 27°C for 48 hr, after which radicle length was measured again. Radicle elongation in the bioassay was statistically analyzed using three-way analysis of variance, the factors being level of juglone applied, moisture, and time.

Experiment 2. An experiment to measure the response of black alder seedlings to juglone and aeration was carried out under ambient temperature conditions and artificial lighting in a laboratory. A randomized design with three juglone levels, two aeration levels, and three replications was employed. A hydroponic medium was chosen rather than a soil medium so that aeration levels could be controlled and to minimize or eliminate microbial degradation and sorption of juglone that normally occur in soil.

The three treatments were a control lacking juglone, 2×10^{-6} M and 2×10^{-5} M juglone. These concentrations were chosen on the basis of the results of a hydroponic study of the sensitivity to juglone of several woody species including black alder (Rietveld, 1983). In this study, 10^{-6} M juglone did not cause a significant reduction in shoot and root dry weight production of black alder, but 10^{-5} M did. Juglone was dissolved in 1 liter of water and added to the nutrient solution.

The air flow rates for the low and high aeration treatments were 5–10 ml/min and 350 ml/min, respectively.

The experiment was conducted on a bench top under a combination of fluorescent and incandescent light, using a 16-hr photoperiod. Depending on the height of the plant, the light intensity at the terminal leaf ranged from 300 to 600 $\mu\text{mol}/\text{m}^2/\text{sec}$ of PAR (photosynthetically active radiation, 400–700 nm in wavelength). The temperature in the room ranged between 10 and 25°C.

Black alder seed was sown in a medium containing peat and Perlite. Thirty-seven days after germination, seedlings were transplanted to 12-cm-deep pots containing sterile sand. Ten cm^3 of complete nutrient solution (Hewitt, 1952) were applied biweekly. After 56 days, all seedlings were inoculated with 0.4 cc packed cell volume (1000g for 15 min) of *Frankia* isolate ArI3 obtained from Berry and Torrey (1979). Twenty-five days later, the seedlings were transferred to aerated 1-liter Erlenmeyer flasks containing an aerated nitrogen-free nutrient solution (Hewitt, 1952). The solution pH was 6.0, and solutions were changed weekly. Quarter-strength Hoagland's solution was used for 16 days, after which full-strength solutions were used. Eighteen and 19 days after transfer to hydro-

ponic culture, pretreatment measurements were made of acetylene reduction and CO₂ evolution. The juglone and aeration treatments were imposed on the following day. Measurements of acetylene reduction and CO₂ evolution were made every day for three days, every second day for eight days, every fourth day for 12 days, and finally 61 days after treatments were started. Bud set in all seedlings occurred between the second to last and last measurement dates.

At each measurement date, acetylene reduction and CO₂ evolution were assessed on two replicates of each treatment. Plants that had the greatest nodule development at the start of the experiment were chosen. Measurements were also made on control plants lacking nodules.

Gas-impervious Saran bags were used for assaying acetylene reduction and CO₂ evolution. A plant was removed from the nutrient solution and the base of the stem was wrapped in Plastilina (modeling clay). A length of hollow glass tubing with a rubber serum stopper attached to the outside end was embedded in the Plastilina to allow gases to be injected and withdrawn from the bag. The root system of the plant was placed inside the bag, and string was drawn tightly around the bag and Plastilina to form a gas-tight seal.

At the start of the assay, air was evacuated from the bag, and 400 ml of air from well-ventilated space in the laboratory was injected using a 50-ml syringe. Injected air samples had CO₂ concentrations similar ($\pm 5\%$) to that of ambient atmospheric air in the autumn in central Illinois. After an incubation of 30 min, duplicate 10-ml samples were withdrawn from the bag and placed in separate Vacutainer tubes (Becton Dickinson, Rutherford, New Jersey) for subsequent analysis of CO₂ concentration. Air in bags without plants was also sampled to determine the ambient CO₂ concentration. Immediately, another 20 ml of air was withdrawn from each bag, and 40 ml of pure acetylene was injected to give a 10% concentration surrounding the root. After an incubation of 1 hr at $20 \pm 1^\circ\text{C}$, duplicate 10-ml samples were withdrawn directly through the wall of the Saran bag and placed in Vacutainer tubes for determination of ethylene and acetylene concentrations.

A gas chromatograph (Hewlett Packard 5890) equipped with flame ionization detector (FID) was used to measure the concentration of ethylene and acetylene. A nickel column 1.5 m long \times 3.175 mm OD was packed with 100–120 mesh Porapak N. Acetylene was used as an internal standard when calculating acetylene-reduction activity (McNabb and Geist, 1979). The same gas chromatograph with a thermal conductivity detector was used to measure the concentration of CO₂ (Dean and Harper, 1986). A stainless-steel column 6 m long \times 3.175 mm OD was packed with 100–120 mesh Porapak N for CO₂ analysis.

Because acetylene reduction and CO₂ evolution were assessed repeatedly on the same plants, it was not possible to determine either nodule or root dry weight at each measurement date. To overcome differences in activity due to

differences in plant size, acetylene-reduction and CO₂-evolution activity are expressed per gram of plant fresh weight at the start of the experiment. Furthermore, acetylene reduction and CO₂ evolution varied widely from one day to the next, although all treatment responses tended to follow the same pattern. Variation in the temperature of the growth room on nights prior to the assays probably caused the fluctuations in plant activity (Table 1) (Dixon and Wheeler, 1983). To eliminate the effects of daily temperature fluctuation on acetylene-reduction and CO₂-evolution rates for the two juglone treatments, values are expressed as a percentage of control plant activity on each particular day.

Before juglone was added, plants randomly chosen for the 2×10^{-6} M treatment had higher acetylene-reduction and CO₂-evolution rates than plants chosen for the 2×10^{-5} M treatment. Therefore, between-treatment comparisons are not valid. The test of least significant difference (LSD) was used to determine whether acetylene reduction and CO₂ evolution of plants within a treatment changed over the period of the experiment relative to the control plants. Aeration level did not detectably influence activity and data were combined to provide a greater sample size.

Acetylene reduction on a nodule dry weight basis was calculated for the final measurement date.

TABLE 1. MEAN ACETYLENE REDUCTION RATE AND CO₂ EVOLUTION RATE \pm STANDARD ERROR OF CONTROL PLANTS (WITHOUT JUGLONE) OVER 63 DAYS^a

Days before (-) and after (+) juglone treatment	Acetylene reduction ($\mu\text{mol/g/hr}$) ^b	CO ₂ evolution ($\mu\text{mol/g/hr}$) ^b
-2	0.136 \pm 0.048	0.0312 \pm 0.0062
-1	0.228 \pm 0.073	0.0416 \pm 0.0022
+1	0.113 \pm 0.036	0.0306 \pm 0.0038
+2	0.147 \pm 0.049	0.0242 \pm 0.0046
+3	0.204 \pm 0.058	0.0198 \pm 0.0018
+5	0.167 \pm 0.049	0.0386 \pm 0.0050
+7	0.136 \pm 0.031	0.0192 \pm 0.0062
+9	0.167 \pm 0.044	0.0394 \pm 0.0036
+11	0.312 \pm 0.072	0.0320 \pm 0.0056
+15	0.150 \pm 0.044	0.0328 \pm 0.0034
+19	0.199 \pm 0.056	0.0216 \pm 0.0030
+23	0.284 \pm 0.064	0.0300 \pm 0.0020
+61	0.149 \pm 0.035	0.0166 \pm 0.0036

^a Means based on four plants.

^b Acetylene reduction and CO₂ evolution expressed on the basis of plant fresh weight at the start of the experiment.

At the start of the aeration and juglone treatments and at the end of the experiment, plant fresh weight was measured. Because of differences in plant size, growth during the experiment is expressed in relative terms. Mean relative growth rate (RGR) is the change in plant size per unit time per unit growing material (Sestak et al., 1971).

RESULTS

Experiment 1. Results of analysis of variance for the bioassay (Table 2) indicate that the effects of juglone treatment and time were highly significant ($P < 0.0001$). Radicle elongation of alder germinants exposed to soil dosed with 10^{-3} M juglone averaged 7.9 mm, significantly ($P < 0.05$) less than mean values for radicles exposed to soil dosed with 10^{-4} M (8.4 mm) or no juglone (8.7 mm). At each time period, root elongation was significantly different, although the response pattern cannot be interpreted in terms of an effect of treatments over time. The alder germinants grew least in treated soil at time 1 and grew best at time 2; times 3 and 4 had intermediate values for radicle elongation. Differences in the soil moisture regime apparently did not affect juglone's ability to inhibit radicle elongation.

The response of alder germinants to different juglone levels with increasing time following dosing is illustrated in Figure 1. In the bioassay four days after treatments were started, radicle elongation in soil dosed with 10^{-3} M juglone was significantly ($P < 0.05$) less than in soils dosed with 10^{-4} M juglone or the control treatment. By day 22, radicle elongation in the 10^{-3} M treatment was again significantly smaller than those in the control, although the difference was much less marked. The bioassay on day 37 showed that radicle elongation

TABLE 2. ANALYSIS OF VARIANCE OF RADICLE ELONGATION OF BLACK ALDER AMONG LEVELS OF JUGLONE AND MOISTURE, AND BY TIME

Source	df	Sums of squares	F	Pr > F ¹
Juglone	2	88	9.94	0.0001
Moisture	1	2	0.54	0.4638
Time	3	813	61.19	0.0001
Juglone × moisture	2	3	0.34	0.7118
Juglone × time	6	194	7.31	0.0001
Moisture × time	3	8	0.60	0.6155
Juglone × moisture × time	6	32	1.22	0.2952
Error	771	3415		

¹Probability under the null hypothesis of all F values that are as extreme as the observed F value.

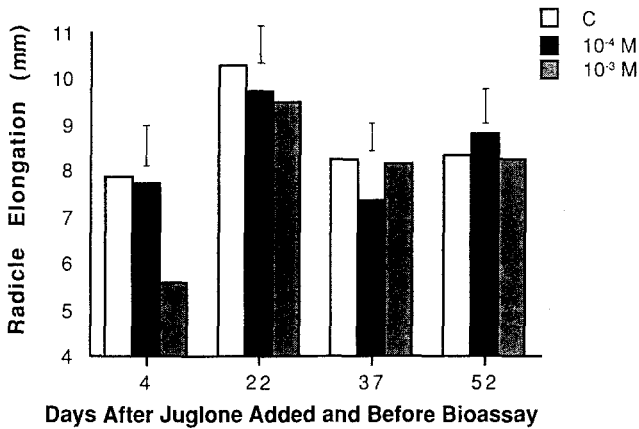


FIG. 1. Mean radicle elongation of black alder germinants exposed to soil incubated with different initial juglone concentrations and measured at four time intervals.

in the control and 10⁻³ M treatments did not differ, although seedlings in the 10⁻⁴ M grew less. There were no differences on day 52.

Experiment 2. There was a significant ($P < 0.0005$) effect of juglone on the relative rate of plant fresh weight accumulation over the period of the experiment (Table 3). Seedlings grown in 2×10^{-5} M juglone had a growth rate of -0.014 g/g/week, significantly less than seedlings in 2×10^{-6} M juglone and the control, which had rates of 0.042 and 0.047 g/g/week, respectively. The aeration treatments and the interaction between juglone and aeration levels did not significantly affect relative growth rates of alder seedlings.

The acetylene-reduction activity of seedlings (Figure 2) decreased after five days of exposure to both 2×10^{-5} M and 2×10^{-6} M juglone. Activity remained low in these plants throughout the rest of the experiment. For example, prior to treatment, seedlings that were subsequently exposed to 2×10^{-5}

TABLE 3. ANALYSIS OF VARIANCE OF RELATIVE GROWTH RATE (PLANT FRESH WEIGHT BASIS) AMONG JUGLONE AND AERATION TREATMENTS

Source	df	Sums of squares	F	Pr > F ¹
Juglone	2	0.01387	15.19	0.0005
Aeration	1	0.00072	1.58	0.2321
Juglone × aeration	2	0.00170	1.86	0.1985
Error	12	0.00548		

¹ Probability under the null hypothesis of all F values that are as extreme as the observed F value.

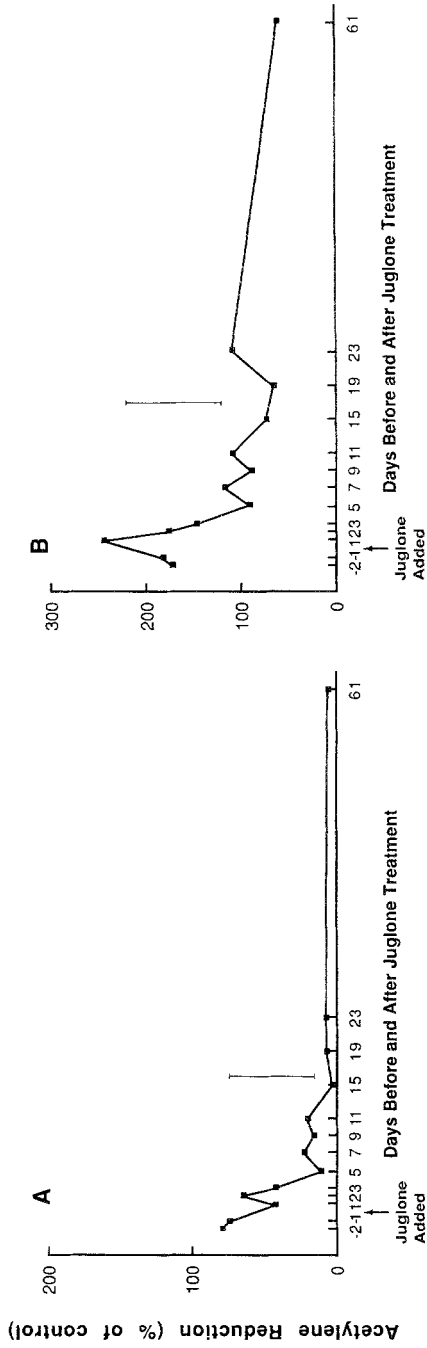


FIG. 2. Rate of acetylene reduction of black alder seedlings treated with 2×10^{-5} M juglone (A) and 2×10^{-6} M juglone (B) expressed as a percentage of the control acetylene reduction rate. Bars are LSD ($P < 0.05$).

M juglone had acetylene-reduction rates 79 and 74% that of control seedlings. After five days of exposure to juglone, the rate had declined to 11% of the mean control rate and remained depressed for the rest of the experiment, yielding a final value 5% that of the control. Seedlings grown in 2×10^{-6} M juglone solutions had pretreatment rates 173 and 182% that of the control mean, and after five days, the rate had declined to 90% of the average value for control seedlings. At the final measurement date, these seedlings were reducing acetylene at a rate 61% that of control seedlings.

When expressed on a nodule dry weight basis, plants in the control, 2×10^{-6} M, and 2×10^{-5} M treatments had acetylene-reduction rates of 32.58, 23.10, and $3.46 \mu\text{mol/g}$ (dry weight)/hr, respectively, at the final measurement date.

The relative rate of CO_2 evolution of seedlings in the two juglone treatments is shown in Figure 3. In the 2×10^{-5} M juglone treatment, seedlings had pretreatment CO_2 -evolution rates 119 and 112% that of control seedlings. After one day of exposure to juglone, the rate had declined to 76% that of control seedlings, and after seven days to 45% of the rate of control seedlings. From day 11 onward, the specific rate of CO_2 evolution relative to that of control plants steadily increased, yielding a final rate of 109% that of control seedlings after 61 days. There was much fluctuation in CO_2 evolution in the 2×10^{-6} M juglone treatment, and no pattern could be discerned.

DISCUSSION

The results of the bioassay of soils dosed with juglone differ markedly from those reported by Fisher (1978). In his study, using a Brant sandy loam, red pine (*Pinus resinosa* Ait.) seedlings subjected to different soil moisture regimes were inhibited by 50 ppm of juglone on an air-dry soil weight basis. In the drier soil, inhibition declined over time but was still apparent after 45 days, while in the wetter soil, inhibition also declined over time, but was still apparent after 90 days. Fisher proposed that the decrease in juglone toxicity over time was due primarily to microbial degradation rather than soil sorption, and microbial activity could be affected by the moisture regime of the soil.

In our study, 10^{-3} M juglone (equivalent to a mean of 45 ppm by soil air-dry weight, and therefore similar to the treatment concentration in Fisher's study) ceased to inhibit radicle elongation between 22 and 37 days after treatments began and 10^{-4} M juglone had no effect. Moreover, the wet and dry treatments had no effect on the seedling response. The more rapid detoxification of juglone in our study compared to that in Fisher's study may be due to the different soils used for the bioassay. Fisher used a fine sandy loam that had been planted to pine, whereas our soil was an organically rich, prairie-derived soil

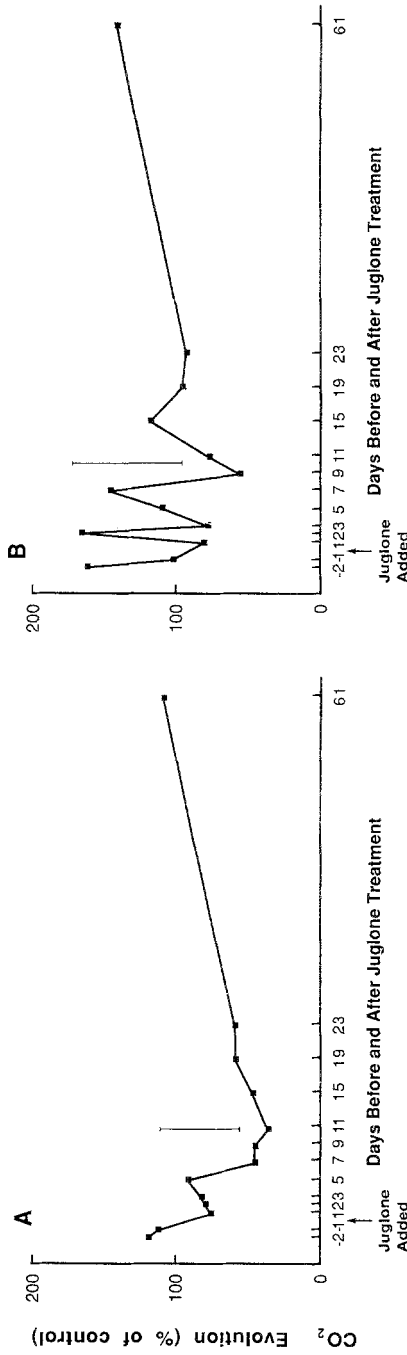


FIG. 3. Rate of CO₂ evolution of black alder roots treated with 2 × 10⁻⁵ M juglone (A) and 2 × 10⁻⁶ M juglone (B) expressed as a percentage of the control CO₂ evolution rate. Bars are LSD (P < 0.05).

that is used extensively for crop production. Native black walnut trees occurred close to the abandoned agricultural field from which our soil samples were taken. Microbial activity would probably be higher in the rich prairie soil, and therefore breakdown of juglone would be more rapid. Alternatively, juglone may be adsorbed more readily in a soil high in organic matter (Ponder, 1987). The absence of a moisture effect in our bioassay indicates that soil sorption may be more important than microbial degradation in detoxifying juglone in the rich prairie soil, or that juglone is subject to anaerobic attack by microbes (Schmidt, 1988).

An attempt was made to quantify the concentration of juglone in the soil at the end of each bioassay using a chloroform extraction technique (Ponder and Tadros, 1985) together with gas chromatography (FID and a 25-m fused capillary column). This proved unsuccessful due to the high diversity and quantity of soil organic materials that apparently prevented the segregation of juglone peaks in the chromatographs.

The hydroponic experiment, by eliminating the effects of soil microbial degradation and soil sorption of juglone, allowed us to measure the direct effect of juglone on plant growth, acetylene reduction, and root respiration without the complicating factors inherent in soils. Rietveld (1983) found that a concentration of 10^{-6} M juglone did not significantly affect the root and shoot growth of black alder in hydroponic culture, but that 10^{-5} M juglone caused a significant inhibition. In our study, 2×10^{-5} M juglone also caused a significant reduction in growth; however, 2×10^{-6} M juglone did not affect growth, confirming Rietveld's findings.

Prior to this work, there had been no studies of the effect of juglone on nitrogen fixation of plant species that have *Frankia* as their symbiont; however, the growth of *Frankia* cultures in vitro has been shown to decrease as juglone concentration increases (Dawson et al., 1981; Dawson and Seymour, 1983). Vogel and Dawson (1985) found that the in vitro growth of four of five *Frankia* isolates was inhibited by 10^{-5} M juglone and that all isolates were inhibited by 10^{-4} M juglone. In addition, nodulation of black alder in soil by *Frankia* isolate ArI3 was reduced at juglone concentrations of 10^{-3} and 10^{-4} M. In our study, the same *Frankia* isolate ArI3 was used to nodulate the black alder seedlings. Acetylene reduction activity in both the 2×10^{-5} and 2×10^{-6} M juglone treatments was reduced in vivo. The reduction in respiration reported in this experiment may explain black alder's reduced nitrogenase activity as estimated by acetylene reduction, since nitrogen fixation is an energy-demanding process.

Black alder is generally regarded as a species that improves the nitrogen fertility of soils (Dawson et al., 1983; Rietveld et al., 1983) via the incorporation of fixed nitrogen in sloughed root tissue and litter into the soil. In a study of juglone allelopathy, total soil nitrogen to a depth of 61 cm under black alder-black walnut interplantings, in which alder was visibly injured, and under wal-

nut alone, did not differ (Ponder, 1987). Perhaps juglone in soil under the mixed planting could have both directly suppressed the ability of black alder to fix atmospheric nitrogen and indirectly suppressed nitrogen fixation by damaging the host plant.

The level of oxygen in the hydroponic solution did not significantly affect the parameters measured in this experiment. It is possible that an airflow rate of 5–10 ml/min was sufficient to satisfy the oxygen demand of the seedling roots. It is also possible that two replications may have been insufficient to detect differences due to aeration.

The rate of root respiration (measured as CO₂ evolution) in seedlings in the 2×10^{-5} M treatment initially declined relative to that in the control treatment. Juglone has been shown to inhibit the oxygen uptake by leaves of tomato and bean plants at concentrations above 10^{-5} M (Perry, 1967). Köeppe (1972) suggested that juglone-induced reduction of respiration is due to the inhibition of some of the coupled intermediates of oxidative phosphorylation, resulting in a slowing of electron flow to O₂ (i.e., an affect on aerobic respiration). It is possible that the initial decline in relative CO₂ evolution in the juglone-treated black alder seedlings was due to inhibition of aerobic respiration. Subsequent increases in CO₂ evolution per unit of initial plant fresh weight relative to the control respiration rate may be due to a partial recovery of respiratory activity after prolonged exposure to juglone. It is more likely that the increase in respiration of roots exposed to juglone relative to root respiration of control seedlings is a function of the decline in respiration of the control seedlings reflected in Table 1.

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HOST PLANT IRIDOID-BASED CHEMICAL DEFENSE OF AN APHID, *Acyrtosiphon nipponicus*, AGAINST LADYBIRD BEETLES

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Abstract—A Rubiaceae-feeding aphid, *Acyrtosiphon nipponicus*, is seldom attacked by the ladybird beetle, *Harmonia axyridis*. A potent deterrent against the beetle was isolated from the aphid and identified as paederoside, an iridoid glycoside originating in the aphid's host, *Paederia scandens*. The iridoid content was as high as 2% of the intact body weight, and a large portion was found in the cornicle secretion.

Key Words—Defense, deterrent, sequestration, iridoid glycoside, paederoside, *Acyrtosiphon nipponicus*, Homoptera, aphid, Aphididae, *Harmonia axyridis*, Coleoptera, Coccinellidae, *Paederia scandens*.

INTRODUCTION

A ladybird beetle, *Harmonia axyridis* (Pallas), feeds on a variety of aphids during its larval and adult stages. However, a monophagous aphid, *Acyrtosiphon nipponicus* (Essig et Kuwana) (synonym: *Aulacophora paederia* Takahashi), which feeds exclusively on *Paederia scandens* Merrill (Rubiaceae), is seldom attacked by the ladybirds. When a hungry adult of *H. axyridis* bites into the aphid, it immediately drops the aphid, salivates, and quickly escapes from the aphid colony. Usually this is accompanied by a persistent grooming of its mouthparts. The aphid frequently secretes droplets from a pair of cornicles (Figure 1) when attacked by ladybirds and quickly smears the fluid onto the predator's mouthparts. The cornicle fluid strongly deters feeding of the ladybirds. Such feeding deterrence in the ladybirds was found to be due to a chemical

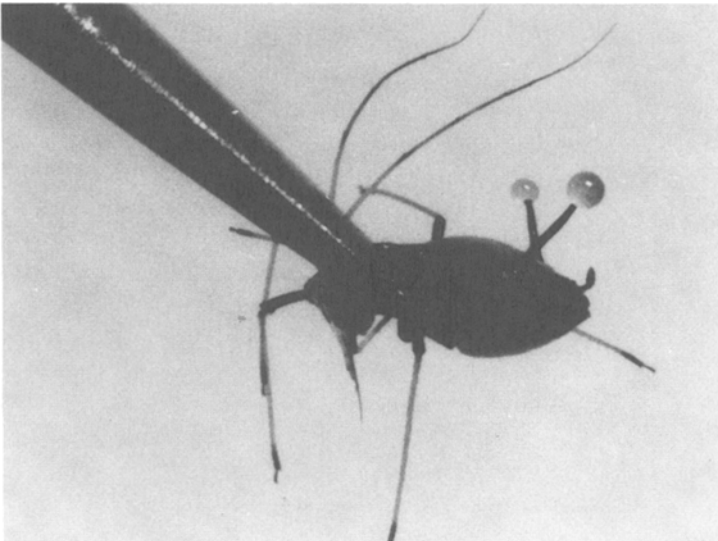


FIG. 1. *Acyrthosiphon nipponicus* secreting the cornicle fluid after disturbance. Usually the aphid quickly smears the fluid onto the forceps if picked up gently. The droplets contain paederoside in high concentration.

sequestered by the aphid from its host plant, *P. scandens*. We describe here the isolation and identification of the feeding deterrent of *A. nipponicus* against *H. axyridis*.

METHODS AND MATERIALS

Bioassay. Pupae of *H. axyridis* were collected from the field in Kyoto City. After adult ecdysis, they were regularly fed with drone honeybee powder (Matsuka and Nijima, 1985) and water for at least three days and then used for feeding bioassay. An aphid dummy similar in size to an aphid was made by dipping the round tip (2 mm id) of the glass rod in hot sucrose-agar solution (12% sucrose and 3% agar). *H. axyridis* adults usually feed on the dummy more than 5 sec (Figure 2). When the dummy surface was coated with an extract of *A. nipponicus*, the ladybirds stopped feeding immediately after the first bite. The test samples of known concentration were applied on the dummy surface as a 1- μ l solution (in acetone or methanol) and brought into contact with the predator's mouthparts after evaporating the solvent in an air current. The bioassay was carried out at about 25°C (16:8 light-dark photoperiod) in a chamber (50 × 50 × 50 mm) which is open in front and illuminated by a fluorescent

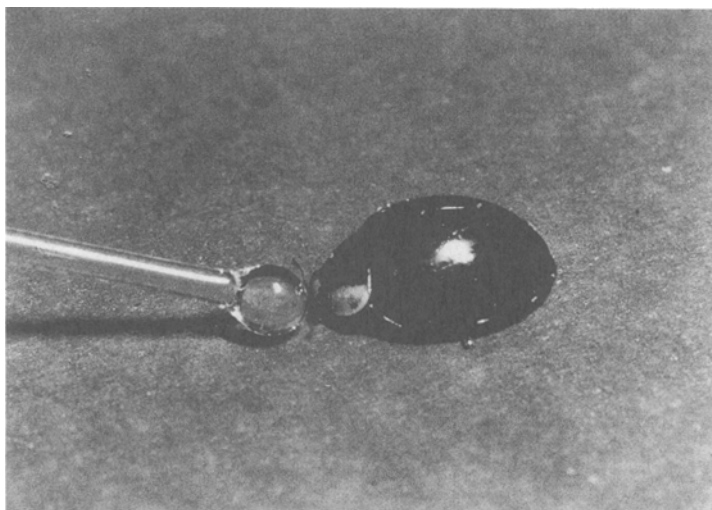


FIG. 2. Feeding deterrence bioassay against *Harmonia axyridis* using an aphid dummy.

lamp (15 W) placed behind a screen at the rear. The deterreny of each sample was obtained as a percentage of the number of ladybirds that stopped feeding on the sample dummy within 5 sec of exposure after the first bite was observed.

Instruments. Optical rotatory dispersion spectra were measured with a Jasco ORD J-5 spectropolarimeter at 30°C, and the ultraviolet (UV) spectrum was recorded with a Shimadzu UV-360 recording spectrophotometer. Field desorption mass spectrum was measured with a Hitachi M-80 mass spectrometer using a carbon emitter at 5.5 kV. Proton and carbon-13 magnetic resonance (PMR and CMR) spectra were measured with a JEOL JNM FX-200 spectrometer (200 MHz) using tetramethylsilane for CD₃OD or sodium 3-trimethylsilyl-1-propane sulfonate for D₂O solvent, respectively, as the internal standards. The letters s, d, t, q, and m represent singlet, doublet, triplet, quartet, and multiplet, respectively (*J*-values in Hz).

Isolation of Compound I (Paederoside). *A. nipponicus* feeding on the vine of *P. scandens* was collected in the campus of Kyoto University during July 1982. The aphids were homogenized in acetone (10 ml), and the residue was extracted twice with acetone (10 ml × 2). The combined extracts (0.20 g), after filtration (Toyo Filter Paper, qualitative No. 2) and evaporation, were fractionated into hexane, ethyl acetate, and water layers by solvent extraction (hexane layer = 47 mg, ethyl acetate layer = 4 mg, water layer = 143 mg). The deterrent activity was recovered from the water layer, which was chromatographed on a reverse-phase column (Sep-pak cartridge C₁₈, Waters Associates), by elut-

ing successively with each 4 ml of water, 20% methanol, 40% methanol, 60% methanol, and 100% methanol (Figure 3). The 20% methanol eluate (24 mg) was subjected in part to high-performance liquid chromatography (HPLC) with a reverse-phase column (Radial Pak liquid chromatography cartridge, μ Bondapak C₁₈, 10 μ m, 100 mm \times 8 mm ID, Waters Associates) eluting with 30% methanol (2 ml/min). The eluate was fractionated into eight fractions, monitored by refractive index (differential refractometer, model R 401, Waters Associates) as shown in Figure 4A. Pure compound I, isolated from fraction 6 [retention time (*Rt*) = 12.0 min, yield = 20 mg from 1.3 g of the intact aphids], was then crystallized from acetone to yield colorless needles (mp 118°C). $[\alpha]_D = -44^\circ$, $[\alpha]_{233} = -3720^\circ$ ($c = 0.36$, methanol). UV: $\lambda_{\max} = 233$ nm ($\epsilon = 11,500$, methanol). PMR (CD₃OD): δ 7.30 (1H, d, $J = 2.2$), 5.94 (1H, d, $J = 1.8$), 5.73 (1H, m), 5.56 (1H, broad d, $J = 7$), 4.85 (2H, m), 4.68 (1H, d, $J = 8.0$), 3.92 (1H, double d, $J = 12.0$ and 1.9), 3.74 (2H, m), 3.19 (1H, double d, $J = 8.0$ and 8.5), 2.34 (3H, s).

Isolation of Compound III (Methyl Paederosidate). Compound III was isolated as an artifact when methanol was used for extraction instead of acetone. The compound was eluted with 40% methanol on a Sep-pak cartridge C₁₈, and purified by preparative HPLC (*Rt* = 39.6 min, under the same condition for compound I). $[\alpha]_D = +13^\circ$, $[\alpha]_{271} = +680^\circ$ ($c = 1.67$, methanol). PMR (CD₃OD): δ 7.66 (1H, d, $J = 1.2$), 6.02 (1H, m), 5.10 (1H, broad d, $J = 15$), 5.06 (1H, d, $J = 8.4$), 4.94 (1H, broad d, $J = 15$), 4.72 (1H, d, $J = 7.6$), 3.86 (1H, double d, $J = 12.0$ and 1.2), 3.74 (3H, s), 3.62 (1H, double d, $J = 12.0$ and 6.0), 3.04 (1H, m, $J = 7.8$, 6.0, and 1.2), 2.63 (1H, double d, $J = 8.4$ and 7.8), 2.34 (3H, s). CMR (D₂O): δ (off-resonance C-H splitting pattern) 15.64 (q), 42.81 (d), 47.24 (d), 54.61 (q), 63.61 (t), 68.09 (t), 72.27 (d), 72.58

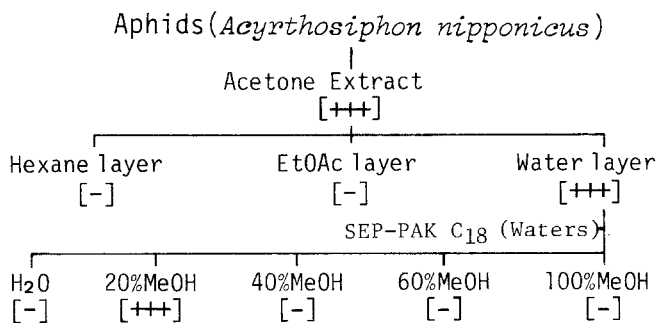


FIG. 3. Separation scheme of the aphid extract and the deterrent activity of each fraction; [−]: less than 10%, and [+++]: more than 90% detergency were observed at a dose of 300 μ g aphid equivalent/dummy ($N = 20$).

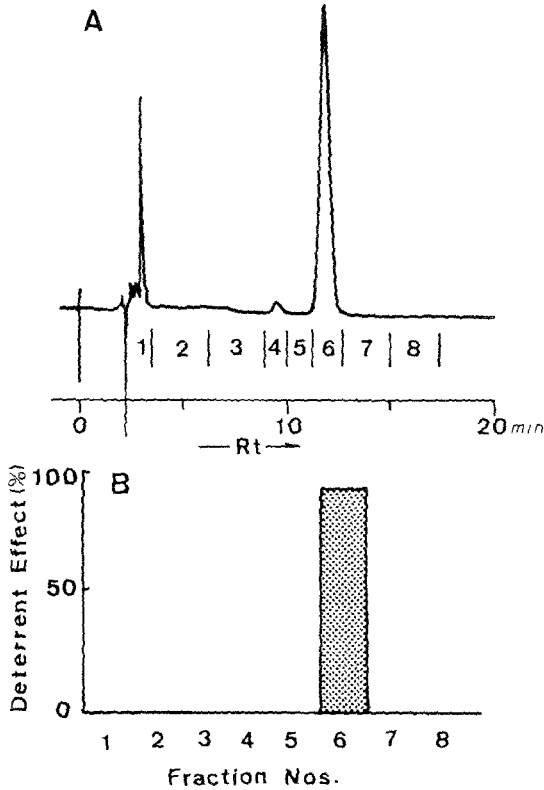


FIG. 4. (A) Liquid chromatogram of the 20% methanol eluate using refractive index. (B) Deterrent activity of each fraction against *Harmonia axyridis* at a dose of 300 μg aphid equivalent/dummy ($N = 20$).

(d), 76.74 (d), 78.40 (d), 78.89 (d), 101.70 (d), 103.02 (d), 109.27 (s), 134.39 (d), 146.31 (s), 157.72 (d), 172.37 (s), 176.53 (s).

RESULTS

Deterreny of Aphid Extracts. The deterrent component of *A. nipponicus* against the ladybirds was efficiently extracted with acetone. The crude acetone extract at various doses was tested on *H. axyridis* adults using the aphid dummy in the manner as shown in Figure 2. Figure 5 shows the dose-response curve of the crude extract. The mean deterreny at the 50% dose-response was approximately 50 μg aphid equivalent per dummy.

Isolation and Identification. An acetone extract of *A. nipponicus* was frac-

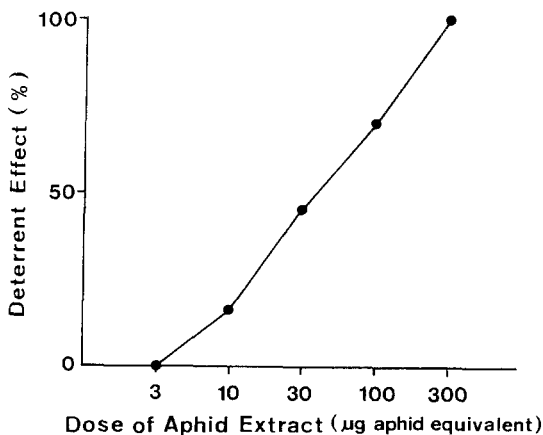


Fig. 5. Dose-response curve of the acetone extract of *Acyrthosiphon nipponicus* in the feeding test using *Harmonia axyridis* ($N = 30$).

tionated into hexane, ethyl acetate, and water layers by solvent extraction. The water layer showed the activity, which was chromatographed on a C_{18} reverse-phase cartridge column. The deterrent activity was recovered from a fraction eluted with 20% methanol (Figure 3). The active fraction was then subjected to preparative HPLC (Figure 4A), and the deterrent activity was found solely in fraction 6 (Figure 4B). The active compound I was isolated as colorless crystals (mp 118°C) from fraction 6. The yield of compound I was 15–20 mg from 1 g of intact aphids.

Compound I was characterized as paederoside, an iridoid glucoside known from aphid's host plant, *P. scandens* (Inouye et al., 1968, 1969), the structure of which has been revised by Kapadia et al. (1979). Field desorption mass spectrometry was employed in order to obtain an unequivocal assignment of the molecular weight (M : m/z 446), in which prominent peaks m/z 447 ($M+H$) and 469 ($M+Na$) were observed along with other fragment peaks, as shown in Figure 6. The dose-response of authentic paederoside provided by Dr. H. Inouye (from *P. scandens*) also coincided with that of compound I, as shown in Figure 7. The mean detergency at the 50% dose-response was approximately $1\ \mu\text{g}/\text{dummy}$, comparable to detergency of the original aphid extract (see Figure 4, a $50\text{-}\mu\text{g}$ aphid equivalent extract contains approximately $1\ \mu\text{g}$ of paederoside).

Activity of Iridoid Analogs. Paederoside is a tricyclic iridoid with an unique thiocarbonate group, OCOSCH_3 , which might be indispensable for detergency. However, asperuloside (II), an acetate analog of the iridoid glucoside, also

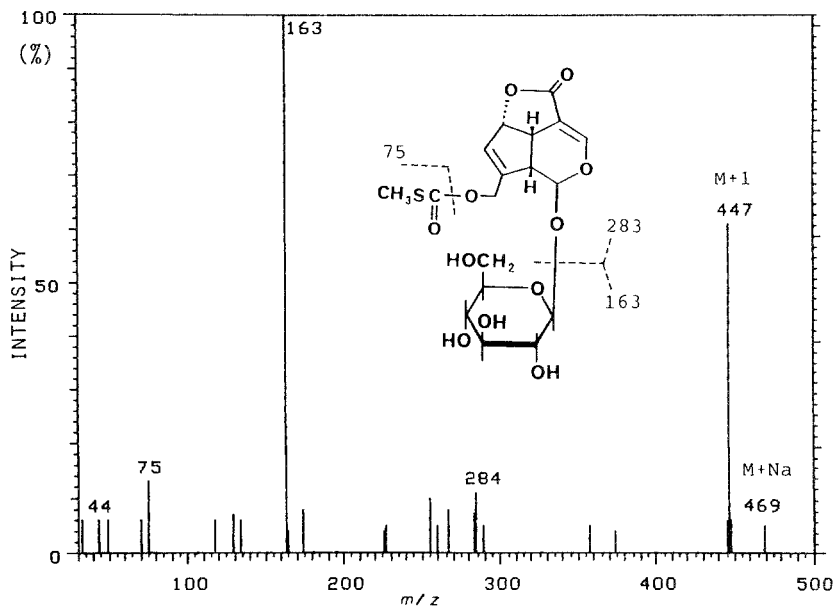


FIG. 6. Field desorption mass spectrum of paederoside (I).

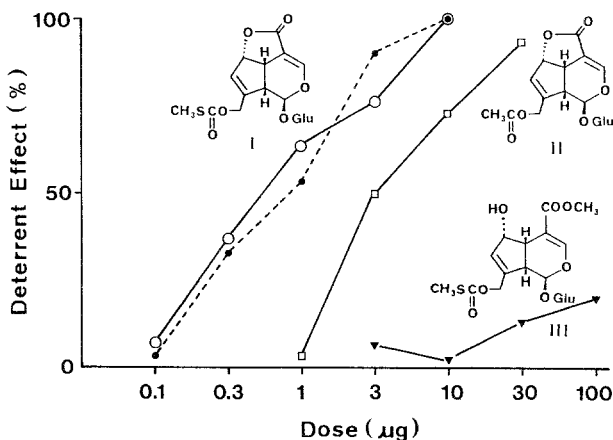


FIG. 7. Dose-response of paederoside (I) (solid line: from aphids, *Acyrtosiphon nipponicus*; broken line: from the host plant, *Paederia scandens*), asperuloside (II) and methyl paederosidate (III) in the feeding deterrence bioassay using *Harmonia axyridis* ($N = 30$).

deterred the ladybirds, while methyl paederosidate (III), a bicyclic analog with the sulfur-containing moiety, was shown to be inactive (Figure 7).

DISCUSSION

It has been clearly demonstrated that *A. nipponicus* is chemically protected from the predatory attack of a ladybird beetle, *H. axyridis*, by possessing a potent deterrent, paederoside (I), which originates from its host plant, *P. scandens*. Paederoside is an iridoid glucoside with a methyl-thiocarbonate moiety that is a very rare functional group in nature. It is suggested that methyl mercaptan may be formed through enzymatic hydrolysis of paederoside when the ladybirds contact the compound with their mouthparts. According to our preliminary observations, a dilute methanolic solution of methyl mercaptan repelled the ladybirds when applied as an air puff. However, an analogous compound III was inactive, whereas the acetyl derivative II was moderately deterrent against *H. axyridis*, suggesting the importance of the tricyclic structure rather than the thiocarbonate moiety. Although the host plant contains a few other iridoid glucosides, including asperuloside (II) (Inouye et al., 1968, 1969), the aphid appears to selectively sequester paederoside in the body tissues. The iridoid content was as high as 2% of the intact body weight of the aphid. Microanalysis of the cornicle fluid revealed that the water-soluble material was pure paederoside (approximately 1 $\mu\text{g}/\text{droplet}$). The aphid appears to promote its chances of surviving because of the deterrent effectiveness of the secreted droplets from the cornicles in response to predatory attacks. Similarly, a nymphalid butterfly, *Euphydryas anicia*, which feeds on *Besseyea* and *Castilleja* plants (Scrophulariaceae), is known to sequester iridoid glycosides (catalpol, aucubin, and macfadienoside) in the body tissues (Stermitz et al., 1986). Catalpol was also shown to be sequestered by two *Penstemon*-feeding geometrid larvae (*Meris alticola* and *Nepterpes graefaria*) (Stermitz et al., 1988).

Two other aphid species are known to accumulate host-plant products after feeding. *Aphis nerii* Fonscolombe sequesters cardiac glycosides from *Nerium oleander* (Rothschild et al., 1970), and *Aphis cytisorum* sequesters sparteine and some other analogous compounds from its host plant, *Cytisus scoparius* (L.) (Wink et al., 1982). Interestingly, nepetalactone and nepetalactol, two monoterpenes that are very stimulatory for cats and have been previously identified in *Nepeta* spp., have recently been characterized as the sex attractant pheromones of the vetch aphid, *Megoura viciae* (Dawson et al., 1987). However, in this case, the terpenes may not be directly derived from the aphid host plant, as is the case for other insects that produce iridoid pheromones and allomones (Blum and Hermann, 1978; Rowell-Rahier and Pasteels, 1986).

Iridoids are widely distributed secondary metabolites in the plant kingdom

and are considered to act as chemical barriers against herbivores. *A. nipponicus* seems to have gained an ecological advantage by selectively feeding on and sequestering the specific deterrent iridoid from its host plant, *P. scandens*.

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LEEK ODOR ANALYSIS BY GAS CHROMATOGRAPHY
AND IDENTIFICATION OF THE MOST ACTIVE
SUBSTANCE FOR THE LEEK MOTH,
Acrolepiopsis assectella

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Abstract—When crushed, the leek, *Allium porrum* emits propyl propanethiosulfinate. The unstable thiosulfinate decomposes during GC analysis in numerous compounds, except on very short columns. The propyl propanethiosulfinate is the most attractive substance for the leek moth, *Acrolepiopsis assectella*. This thiosulfinate is not active on *Plutella xylostella* or *Plodia interpunctella* and seems to be repulsive on *Ephestia kuehniella*.

Key Words—*Allium porrum*, leek moth *Acrolepiopsis assectella*, *Plodia interpunctella*, *Ephestia kuehniella*, *Plutella xylostella*, Lepidoptera, Hyponomeutoidea, propyl propanethiosulfinate, propyl trisulfide monoxide.

INTRODUCTION

The biosynthesis of several sulfur compounds identified in the odor of *Allium* mainly involves relatively unstable thiosulfates (Boelens et al., 1971). Although preliminary analysis by thin-layer chromatography (TLC) pointed to their presence in leek odor (Auger and Thibout, 1981), no analysis by gas chromatography-mass spectrometry (GC-MS) had yet proven their existence (Schreyen et al., 1976).

We studied the stability of synthetic propyl propanethiosulfinate on gas chromatography columns in order to develop a technique that would avoid its breakdown. The resulting technique was then applied to leek odor (*Allium porrum*, Liliaceae), and the results obtained were compared for attraction of the

leek moth *Acrolepiopsis assectella* (Hyponomeutoidea) both for substances possibly existing in this odor (Lecomte and Thibout, 1981), and for closely related substances (Lecomte et al., 1987).

The attractiveness of these substances has also been studied on two generalist moths, *Ephestia kuehniella* and *Plodia interpunctella*, and on *Plutella xylostella*, specialist of Cruciferae, the odor of which also contains some of the substances studied (Bailey et al., 1961).

METHODS AND MATERIALS

Instrumentation. Analytical gas chromatography (GC) was performed with a Varian 3300 chromatograph equipped with Varian on-column injector, FID, and FPD (sulfur mode) detectors. Fused silica columns (SGE) (2.5 or 25 m \times 0.33 mm ID; $df = 0.5 \mu\text{m}$) coated with BP 20 were used for analysis. The carrier gas was N_2 at a flow rate 1 ml/min. The injector temperature program was $50^\circ\text{C}/\text{min}$ from 70 to 150°C .

Materials. Dipropyl disulfide and propanethiol were supplied by E. Merck (Darmstadt, West Germany). Dipropyl trisulfide was obtained according to Banerji and Kalena (1980). Propyl propanethiosulfinate and dipropyltrisulfide monoxide [Pr-S-S(O)-Pr] were synthesized by oxidizing the disulfide and trisulfide, respectively, with perbenzoic acid (Auger et al., 1985). Propyl propanethiosulfonate was obtained by oxidizing propanethiol with hydrogen peroxide.

The purity of these compounds was examined by NMR and IR spectroscopy (Auger, 1987).

Leek Volatiles, Trapping, Isolation, and Identification. Leek odor was emitted by cutting green leaves (1 kg) of the Malabare cultivar in a closed glass vessel (0.15 m^3), at room temperature.

Head-space volatiles were trapped during 1 hr on a glass cartridge (20 mm \times 4 mm ID) containing 30 mg of Tenax GC (Alltech Associates) 60–80 mesh directly connected to a Gillian LFS 113 pump (250 ml/min).

The trapped volatiles were eluted from the cartridge with 1 ml of peroxide-free diethyl ether (analytical grade) and immediately analyzed.

The volatiles were tentatively identified by comparing their GC retention times under various conditions to those of authentic samples.

Bioassay. The biological activity of some of the identified substances and analogs on the behavior of the four moth species was evaluated in an olfactometer as previously described (Lecomte and Thibout, 1981; Lecomte et al., 1987). Females of the four species were reared in the laboratory under identical conditions and tested once. For each odor, 30 mature females were placed individually in a glass tube (90 cm length \times 2 cm diameter) opposite the air flow (20

cm/sec) entry. The position of each female in one of three equal longitudinal sections was recorded after 1 min of observation in pure air, then after 1 min in air loaded with the odor being tested (10^{-2} mg of product evaporated/min). The χ^2 test was used to compare the distributions obtained in pure air and in odor. Only these values are given here in Table 1. Some previously published results on *A. assectella* (Lecomte et al., 1987) were used here for comparison.

RESULTS

Propyl propanethiosulfinate (90% pure), which contained only dipropyl disulfide by NMR analysis, when chromatographed on a 25-m column, revealed propanethiol, dipropyl disulfide, dipropyl trisulfide, and propyl propanethiosulfonate in varying proportions, depending on the temperature, and only a minimal amount of the injected substance, thus suggesting its decomposition on the column (Figure 1). The shorter the column, the greater the amount of the injected substance observed. On a 2.5-m column, propyl propanethiosulfinate appears to be accompanied only by dipropyl disulfide in the real proportion (90:10) and with no other compound present (Figure 2).

The chromatogram of leek volatiles obtained by using the same short column under identical conditions reveals mainly propyl propanethiosulfinate, although with a significant quantity of disulfide (Figure 3). However, on a 25-m column, under various temperature conditions, only a small quantity of thiosulfinate is found, although the corresponding thiol, disulfide, and trisulfide appear, accompanied by propyl propanethiosulfonate and, in minor quantities, methyl disulfide and trisulfide (Figure 4).

The attractiveness of the different sulfur compounds tested differs according to species (Table 1). In *A. assectella*, the four compounds are highly attrac-

TABLE 1. RESPONSE OF FOUR MOTHS TO LEEK VOLATILES AND ANALOGS^a

	<i>Acrolepiopsis assectella</i>	<i>Plutella xylostella</i>	<i>Ephestia kuehniella</i>	<i>Plodia interpunctella</i>
Dipropyl disulfide	22.89***	5.89	2.62	3.50
Propyl propanethiosulfinate	38.11***	3.34	10.01 ^b **	2.14
Dipropyl trisulfide	19.92***	0.68	2.62	5.20
Dipropyl trisulfide monoxyde	20.36***	0.00	0.08	0.59

^aThe distributions of the females in the olfactometric tube are compared by the χ^2 test in the presence of pure air and substance-loaded air (***, $P < 0.001$; **, $0.001 < P < 0.01$; $2 df$). The χ^2 value is taken as a measure of the difference between the pure air and the odorous air distributions.

^bThe sole repulsive effect observed.

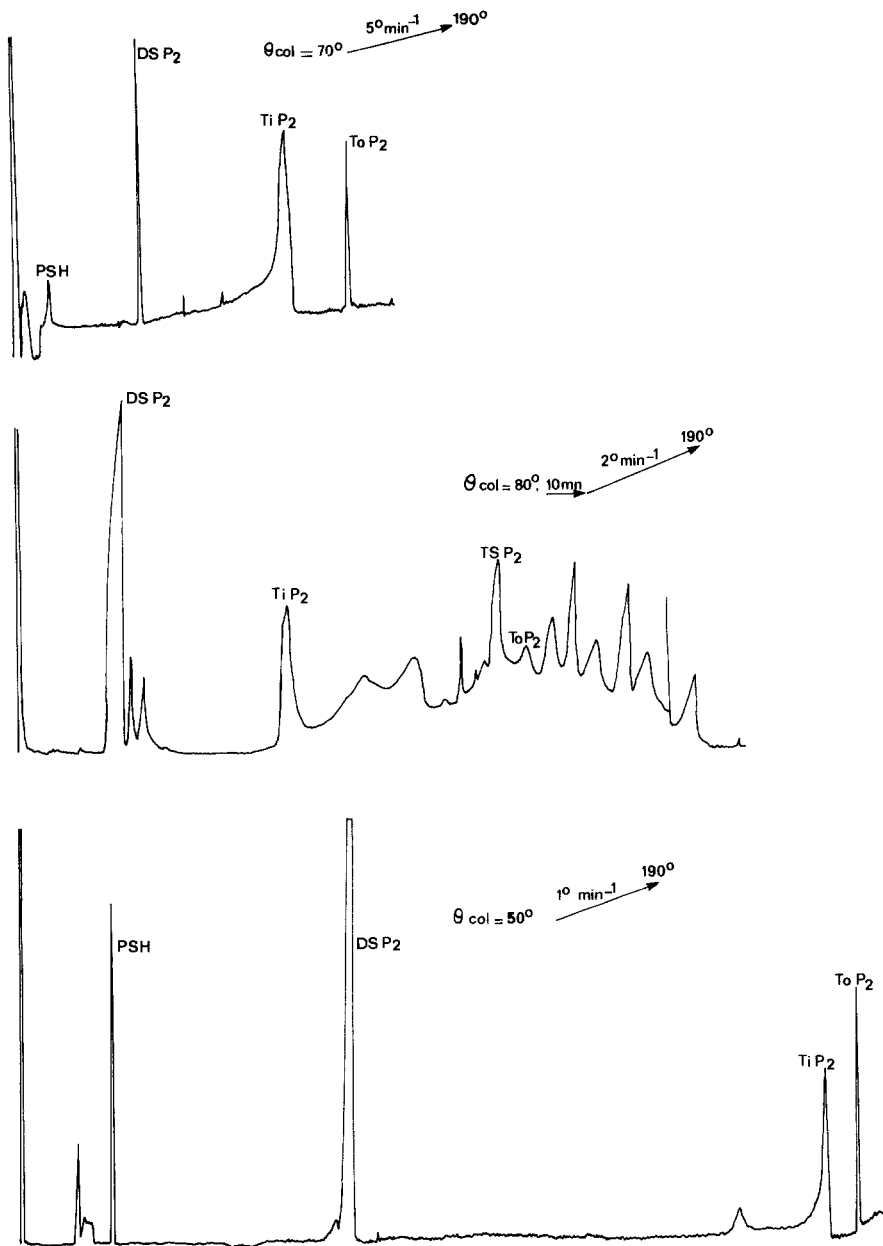


FIG. 1. Gas chromatogram of synthetic propyl propanethiosulfinate (TiP₂) containing 10% of dipropyl disulfide (DS P₂). Column = 25 m × 0.33 mm (ID); various temperature programs. On column injection. FP detector. PSH = propanethiol, ToP₂ = propyl propanethiosulfonate, TSP₂ = dipropyl trisulfide.

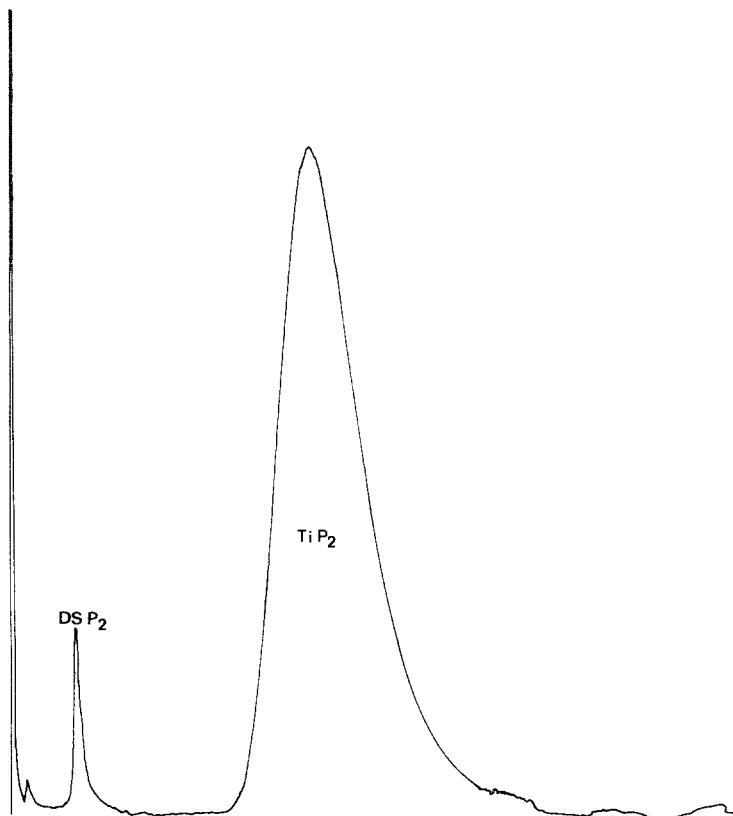


FIG. 2. Gas chromatogram of synthetic propyl propanethiosulfinate (TiP_2) containing 10% of dipropyl disulfide (DS P_2). Column = 2.5 m \times 0.33 mm (ID); temperature program = 70°C for 5 min, then 10°C/min to 100°C. On column injection. FP detector.

tive (highly significant χ^2 values), especially propyl propanethiosulfinate. In *P. xylostella*, dipropyl disulfide may be slightly attractive, while the other compounds are inactive (nonsignificant χ^2 values). In *E. kuehniella* and *P. interpunctella*, the tested compounds are inactive except for propyl propanethiosulfinate, which could act as a repellent of *E. kuehniella*.

DISCUSSION

On long gas chromatographic columns, propyl propanethiosulfinate is broken down, possibly by mechanisms other than simple disproportionation, into dipropyl disulfide since propanethiol and dipropyl trisulfide appear, apart from dipropyl disulfide and propyl propanethiosulfonate. In rapid chromatography

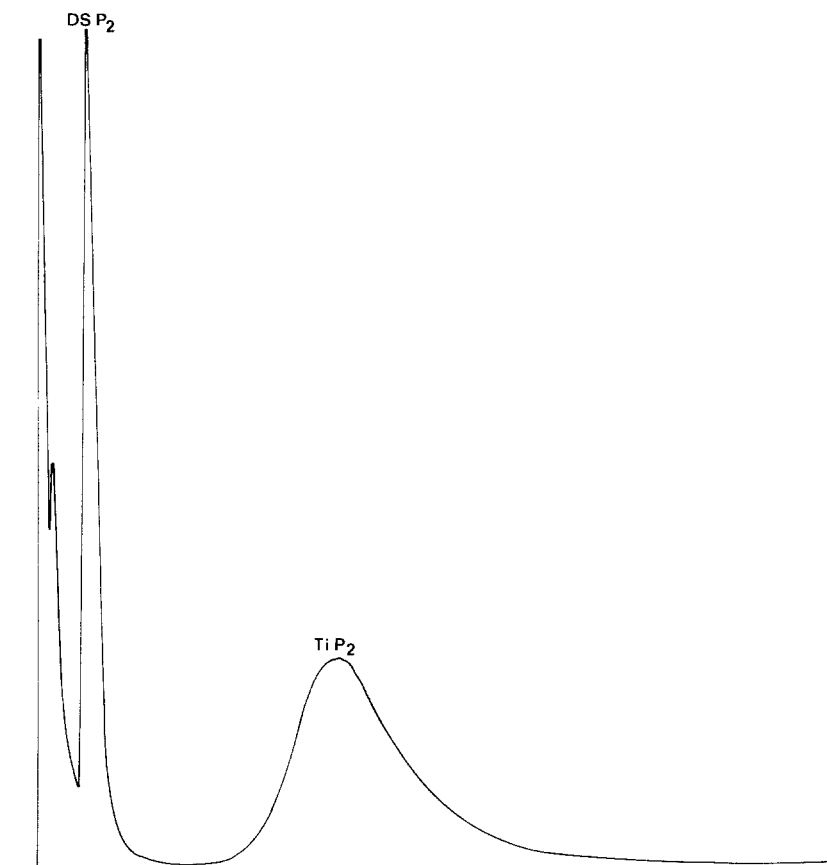


FIG. 3. Gas chromatogram of the leek volatiles, trapped on Tenax. Column = 2.5 m \times 0.33 mm (ID); temperature program = 70°C for 5 min, then 10°C/min to 100°C. On column injection. FP detector. DS P₂ = dipropyl disulfide, TiP₂ = propyl propanethiosulfinate.

(very short column), the thiosulfates do not break down. This technique allows one to conclude that leek odor just emitted contains primarily propyl propanethiosulfinate and perhaps a small amount of dipropyl disulfide. The majority of sulfur volatiles identified by GC-MS in *Allium* spp. are thus artifacts produced during the isolation of the sample and during chromatography.

It has been shown previously that sulfur compounds possibly existing in leek odor and their closely related analogs were attractive to the leek moth (Lecomte and Thibout, 1981; Thibout et al., 1982). Propyl propanethiosulfinate

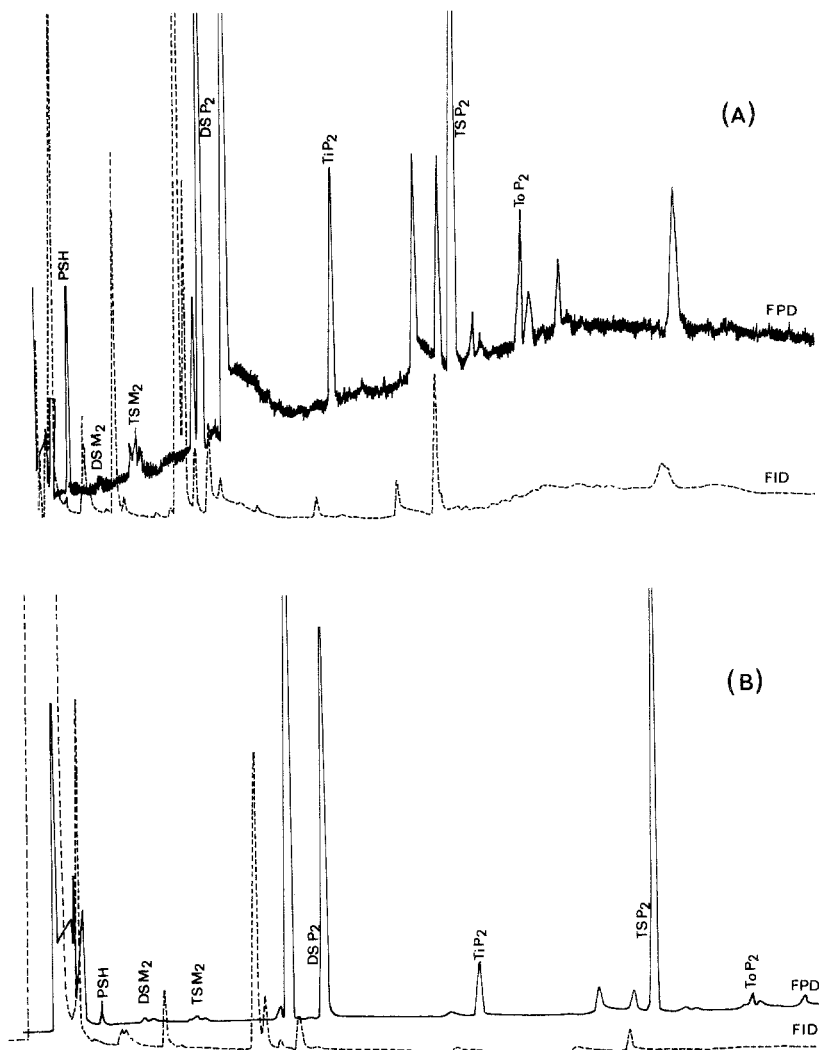


FIG. 4. Gas chromatogram of the leek volatiles, trapped on Tenax. Column = 25 m \times 0.33 mm (ID); temperature program = (A) 2°C/min from 80°C to 150°C, (B) 1°C/min from 80°C to 150°C. On column injection. FP and FI detectors. DS M₂ = dimethyl disulfide, TS M₂ = dimethyl trisulfide; other compounds, see Figure 1.

is, however, more attractive than dipropyl disulfide, while dipropyl trisulfide monoxide is slightly more active than dipropyl trisulfide (Lecomte et al., 1987). Moreover, propyl propanethiosulfinate and other thiosulfonates are more stimulating than disulfides in *A. assectella* during oviposition (Auger, 1987).

These results confirm that the propyl propanethiosulfinate emitted when the leaves were crushed is one of the principal compounds that allow the moth to recognize *Allium* plants, particularly the leek.

The specificity of the action of this compound, unattractive to the other insects tested and even repellent for one of them, is much greater than in the disulfides that follow its breakdown in *Allium* extract and which, unlike thiosulfonates, also exist in other plant genera like Cruciferae. Moreover, these disulfides could be attractive to the adult *P. xylostella* moth, although less so than isothiocyanates, which are specific to Cruciferae (Auger, 1987).

In the light of these results, it seems necessary to reexamine GC analysis of other plant odors. Previously obtained results could be challenged if the identified compounds are produced by the breakdown of less stable precursors during analysis.

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ALLELOPATHIC POTENTIAL OF SORGHUM-SUDANGRASS HYBRID (SUDEX)¹

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Abstract—Experiments were conducted under controlled conditions to investigate the apparent allelopathic effects of sudex [*Sorghum bicolor* (L.) Moench × *Sorghum sudanese* (P.) Stapf. cv. FFR 201] on weed and vegetable species. Allelopathic potential, as measured by radicle elongation of herbaceous indicator species, decreased with increasing sudex age. Greatest potential allelopathic activity of sudex shoot tissue was observed when sudex was collected at 7 days of age. Small-seeded broadleaf species were more inhibited in the presence of sudex shoot tissue than were grass species. Two major phytoinhibitors were isolated from aqueous extracts of sudex shoot material by partitioning with diethyl ether, followed by thin-layer and liquid column chromatography. Phytoinhibitors were identified as *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde, potentially the enzymatic breakdown products of the cyanogenic glycoside dhurrin. The I_{50} values of these compounds using a cress (*Lepidium sativum* L.) seed bioassay were 140 and 113 $\mu\text{g}/\text{ml}$ for the acid and aldehyde, respectively. Sudex tissue collected at 7 days of age possessed a greater percentage of these phytoinhibitors on a per gram basis than did older sudex tissue. As sudex tissue age increased, the percentage of *p*-hydroxybenzaldehyde in ether extracts of tissue also increased, while the percentage of *p*-hydroxybenzoic acid decreased.

Key Words—*Sorghum bicolor* (L.) Moench × *Sorghum sudanese* (P.), Stapf. cv. FFR 201, sudex, allelopathy, shoot tissue, plant age, radicle elongation, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde.

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INTRODUCTION

Cover crops are used to control soil erosion and reduce weed populations in nurseries and in agronomic crops. Between production cycles or seasons, cover crops are also used as a source of green manure for the improvement of soil structure and organic matter content (Martin et al., 1976). Sudex is commonly selected as a summer annual cover crop because of its rapid growth and ability to suppress weed biomass (Forney et al., 1985).

Both sudex and related sorghum species have shown possible allelopathic interactions with horticultural and agronomic species. Iyer et al. (1980) demonstrated that sudex incorporated into a container soil medium inhibited the growth of pine (*Pinus*) seedlings. Forney et al. (1985) obtained excellent suppression of broadleaf weed species when sudex was used as a green manure crop preceding alfalfa (*Medicago sativa* L.) establishment. Forney and Foy (1985) also discovered that root leachates from hydroponically grown sudex inhibited the growth of both monocot and dicot seedlings. Purification of collected sudex phytotoxins was not attempted.

Recently, we have found that the growth of selected woody and herbaceous seedlings was significantly reduced when seedlings were cocultivated with sudex and when sudex leaf material was incorporated into the growth medium (Weston and Geneve, 1987). In further studies with eastern redbud (*Cercis canadensis* L.), growth reductions could not be reversed with increased fertilizer rates. Additionally, redbud growth decreased linearly with increased amounts of fresh or dried sudex incorporated into the growth medium (Geneve and Weston, 1988).

Since sudex shows potential for use as a weed suppressive green manure crop, it is important to determine which stage of plant growth is associated with the greatest allelochemical production. Sorghum (*Sorghum bicolor* L. Moench), a closely related species, has been shown to inhibit germination and development of weed seedlings (Panasuik et al., 1986) and to contain greatest concentrations of the cyanogenic glycoside dhurrin in the seedling stages of growth (Conn, 1981; Haskins et al., 1984). In addition, Lehle and Putnam (1982) also found that Bird-a-Boo sorghum toxicity was greatest in herbage of 2-week-old plants.

Although recent laboratory, greenhouse, and field studies suggest that allelochemicals present in sudex tissue suppress weed growth, the potential phytotoxins have not been characterized. To further explore the possible allelopathic potential of sudex shoot tissue under controlled laboratory conditions, studies were undertaken to: (1) determine the influence of age of sudex tissue upon inhibition of seed germination and radicle elongation of broadleaf and grass species, (2) assess the effect of partitioned sudex shoot extracts on seed germination and radicle elongation, (3) isolate and identify the inhibitors

present in sudex shoot tissue responsible for the reduction in seedling root growth, and (4) determine the influence of age of sudex shoot tissue upon the levels of inhibitor(s) present in shoot extracts.

METHODS AND MATERIALS

Collection of Sudex Material. For all experiments, sudex cv. FFR 201 was seeded directly into plastic flats containing a nonpasteurized soilless growth medium [5 parts Promix BX (Premier Brands) to 1 part perlite, v/v]. Flats were placed in a glasshouse with temperatures maintained at 23–27°C day/20–24°C night. Supplemental lighting was provided by high-pressure sodium vapor lamps with photosynthetic photon flux density of approximately 500 $\mu\text{mol}/\text{sec}/\text{m}^2$ at a plant height of 30 cm. Flats were watered overhead as needed and fertilized with soluble fertilizer (Peter's) 14 N–6.5 P–13.3 K at 200 ppm N at each watering.

Sudex shoot tissue was harvested at several dates in April through July 1986 by cutting shoots 2 cm above the soil surface. In addition, sudex planted on September 29, 1987, was harvested at one, two, three, and four weeks after planting, at 7, 14, 20, and 30 cm in height, respectively. All sudex tissue was placed in a drying oven at 45°C for five to six days prior to analysis or bioassay. A small amount (15 g) of material was chopped into 1.5-cm pieces and saved for soil bioassays (described below). The remainder was ground separately in a Wiley mill (mesh screen size 1 mm). Powdered material was stored in tightly closed glass containers until used for extraction and quantification of sudex phytoinhibitors.

Bioassay of Sudex Shoot Tissue. A modified Parker (1966) bioassay was used for measurement of inhibition of seed germination and radicle elongation. Maury silt loam (Typic Paleudalf, fine, mixed, mesic) was sifted and combined with sand (1 : 1 v/v) and placed in a 45°C oven for seven days until dry. Treated soil (100 g) was placed in 100- × 15-mm² plastic Petri dish and topped with 1.0 g of chopped, dried sudex. A thin layer of the soil mixture (50 g) was spread over the residue and 35 ml of distilled water was added to the dish.

A 9- × 9-cm piece of filter paper (Whatman No. 1) was placed on the soil surface of each dish. Ten seeds of each of two indicator species were uniformly placed in two rows parallel to each other on the surface of the filter paper. Tomato (*Lycopersicon esculentum* Mill., Niagara), Burpee curly garden cress, foxtail millet [*Setaria italica* (L.) Beauv.] and barnyardgrass [*Echinochloa crus galli* (L.) Beauv.] were utilized as bioassay species. Dishes were covered, taped shut, and placed in a vertical position in a germination chamber to encourage downward root growth. Seeds were held in place by pressure of surrounding chamber upon dishes. Dishes were then placed in a 28°C incubator and radicle

length was measured for each seed four days later. Radicle length of nongerminated seeds was assumed to be 0. Treatments consisted of sudex shoot tissue aged 7, 14, 21, and 28 days, and a control consisting of paper toweling (1.0 g) cut into 1.5- × 1.5-cm pieces. Experiments were analyzed separately for each indicator species, as completely randomized designs with four replicates per treatment. Data were subjected to analysis of variance and means separated using Fisher's protected LSD (0.05) test.

Extraction of Sudex Shoot Material. Shoot tissue (400 g) was extracted for 48 hr with 7200 ml of distilled water on an orbital shaker in a 4°C coldroom (Figure 1). The mixture was filtered through four layers of cheesecloth and centrifuged for 20 min at 12,000g to remove particulate material. Finer particulates were removed by vacuum filtration through Whatman No. 4, 1, and 42 filter paper, respectively. The clear, aqueous extract was sequentially parti-

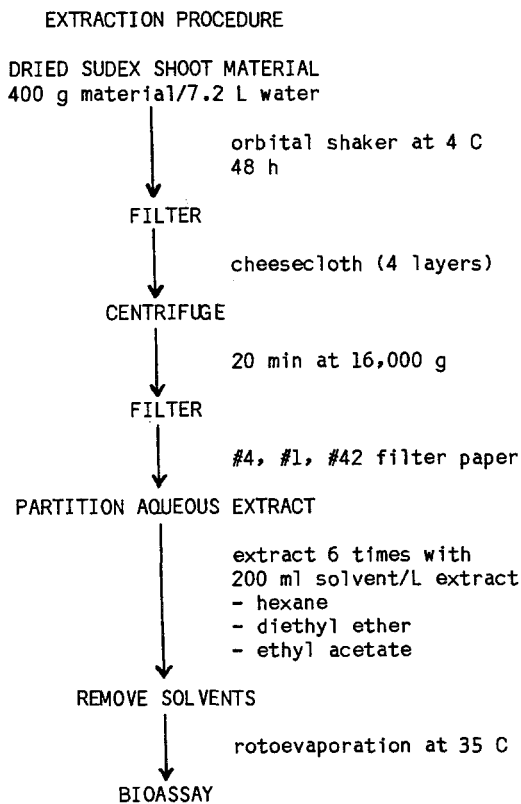


FIG. 1. Flow diagram of the procedure used for extraction and partitioning of dried sudex shoot residues.

tioned with hexane, diethyl ether, and ethyl acetate. Each liter of the aqueous shoot extract was partitioned six times with 200 ml of each of the three solvents. Solvent was removed from each fraction by rotary evaporation at 35°C. The entire process of extraction and partitioning was repeated twice and combined to gather enough material for experimentation.

Each fraction from the solvent extractions was weighed individually and redissolved in chloroform to form solutions at a concentration of 1.0 mg dry residue/ml chloroform. One half milliliter of each fraction was placed in separate glass Petri dishes (60 × 15 mm), which were lined with filter paper (Whatman No. 1). Chloroform was allowed to evaporate from each dish, and 1.5 ml distilled water was added to form a final concentration of 334 μg material/ml. The control consisted of distilled water applied to the filter paper after evaporation of 0.5 ml chloroform. Twenty seeds of Burpee curly cress were then added to each dish. Three replicates of each treatment were arranged in a completely randomized design and placed in a seed germination chamber maintained at approximately 26°C with relative humidity near saturation. Radicle length was measured at 72 hr. Radicle elongation of curly cress was consistently used as a bioassay throughout these experiments because of its sensitivity, uniformity, and availability. Data were subjected to analysis of variance and means were separated by Fishers protected LSD (0.05) test.

Isolation and Characterization of Phytotoxins. The ether extract from above was further separated initially using liquid column chromatography. The extract (380 mg) was loaded onto a silica gel (60 g Baker, 200–250 mesh) flash column (4 cm diameter × 40 cm). Pressure for the column was provided by a laboratory compressed air pump and was regulated to provide a solvent flow rate of 40 ml/min. The column was eluted with the following solvent series: 350 ml chloroform, 200 ml each of 19:1, 9:1 and 5:1 chloroform–methanol, followed by 250 ml methanol. Fractions (20 ml) were collected, examined by TLC (silica gel developed with 7:1 chloroform–methanol), and combined on the basis of similar TLC profile to provide 10 distinct fractions. The 10 fractions were bioassayed separately for inhibitory activity using 250 μg material/dish, or 166.7 μg/ml. Since activity was restricted to two consecutive fractions, these fractions were combined prior to further analysis.

The combined fractions (104.5 mg) were loaded onto a C₁₈ (60 g Baker Flash Octadecyl bulk packing; 40 μm) flash column and eluted as described above using the following solvent series: 350 ml 60:40, 250 ml 75:25, 250 ml 90:10 methanol–water, followed by 250 ml methanol and 250 ml chloroform. Fractions were collected and examined as described above and combined to provide 10 distinct fractions. Bioassay of fractions, using 200 μg material/dish or 133.3 μg/ml, revealed toxicity to be confined to fractions 1–3. These fractions were combined (42.7 mg) and chromatographed via silica gel TLC (Whatman LK5F plates) using a mobile phase of 7:1 chloroform–methanol.

Visualization under UV (254 nm) light revealed six distinct bands; similar bands were scraped off several plates and combined. Plate scrapings were eluted with 3:1 chloroform-methanol through a fritted glass filter, and the eluent from each zone was collected, dried, weighed, and bioassayed at 150 μg material/dish or 100 $\mu\text{g}/\text{ml}$. Strong inhibitory activity occurred in zones 2 ($R_f = 0.35\text{--}0.36$) and 5 ($R_f = 0.57\text{--}0.59$), each of which contained one major component only, visible under UV (254 nm) light. TLC plates were exposed to 5% vanillin in sulfuric acid for colorimetric characterization of active compounds. The migration of materials on silica gel TLC plates in various solvent systems was compared with that of standards of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid (Sigma) as visualized by exposure to UV (254 nm) light and to the vanillin sulfuric acid reagent. The chemical nature and the purity of the two unknown compounds were also examined using high-pressure liquid column chromatography (HPLC) using a Waters Bondapak, radial compression column (8 mm \times 10 cm). The UV detector (Waters LambdaMax model 481) measured absorbance at 254 nm. Solvent used was 50:50 methanol-water at a flow rate of 1.5 ml/min. Identity of compounds was further confirmed by coelution with known standards using TLC and HPLC. Dose-response curves were constructed to determine the concentration of these materials (compounds 1 and 2) required for 50% inhibition of cress seed radicle elongation (I_{50}).

Quantification of Phytotoxins from Sudex Shoots of Various Ages. Sudex shoots of various ages (7, 14, 21, 28 days) were collected and dried as described above. One hundred grams of dried shoot tissue of each age was extracted separately for 48 hr; the four extracts were then sequentially partitioned with hexane and diethyl ether. The diethyl ether extract was dried by rotary evaporation, weighed, and bioassayed as described above at levels of 0, 125, 250, and 500 $\mu\text{g}/\text{dish}$, or 0, 83.4, 166.7, and 333.3 $\mu\text{g}/\text{ml}$. The HPLC system described previously (50:50 methanol-water) was used to separate and quantitate compounds 1 and 2 in each fraction. Standard curves of each purified compound were generated and used to estimate the total amount of compounds 1 and 2 in each sudex tissue extract. This process was repeated, and data represent means of two replications.

RESULTS AND DISCUSSION

Bioassay of Sudex Shoot Tissue. As sudex age increased from 7 to 28 days, allelopathic potential of tissue decreased, as measured by radicle elongation of four indicator species (Table 1). Differential inhibition obtained with sudex tissues of various ages suggests that the concentrations of phytoinhibitors may be greatest in very young sudex tissue and gradually decrease with increasing maturity. Small-seeded broadleaf species were most sensitive to the presence

TABLE 1. INFLUENCE OF AGE OF SUDEX SHOOT RESIDUE UPON RADICLE ELONGATION IN 4 INDICATOR SPECIES AFTER 96 HOURS

Residue age (days)	Radicle length (mm)			
	Curly cress	Tomato	Foxtail millet	Barnyard grass
Control	10.2	9.1	35.8	38.0
7	2.6	0.7	19.5	23.9
14	3.5	2.4	21.2	31.0
21	7.8	7.7	26.4	39.6
28	6.7	7.8	36.4	36.7
LSD (0.05)	2.6	4.3	7.3	8.2

of the sudex tissue, whereas radicle elongation of grass species was less affected by the tissue. This was not unexpected, since several cover crop residues have also shown considerable selectivity in the inhibition of growth of both vegetable and weed seedlings in the field (Putnam and DeFrank, 1983). The modified Parker bioassay is particularly useful for uniform evaluation of tissue toxicity in a soil situation under controlled laboratory conditions. In additional studies performed using pasteurized soil and sudex tissue sterilized by exposure to propylene oxide, the presence of soil microorganisms was not required for the development of sudex tissue toxicity (data not presented). In fact, sudex tissue was generally more inhibitory to seedling indicators under sterile conditions, indicating that soil microorganisms may reduce tissue toxicity over time (Weston and Geneve, 1987). Greenhouse studies have also indicated that sudex toxicity diminishes over a period of eight weeks after incorporation in the growth media (Geneve and Weston, 1988). This may be due, in part, to microbial activity.

Extraction of Sudex Shoot Tissue. Greenhouse-grown tissue was used due to its uniform size obtained under growing conditions and its ready availability. Aqueous extraction of sudex shoot material was performed at 4°C to prevent the breakdown of secondary products by microorganisms. The greatest amount of material was removed from the aqueous shoot extract by partitioning with diethyl ether (Table 2). The greatest inhibitory activity was also associated with the diethyl ether fraction, as measured by the inhibition of cress radicle elongation at 72 hr. The ethyl acetate fraction was also quite inhibitory; however, this fraction was very similar in chemical composition to the diethyl ether fraction when examined using thin-layer chromatography. Very little toxicity remained in the crude aqueous extract after partitioning against the solvent series. Since the greatest toxicity and a comparatively large weight of material were concentrated in the ether fraction, the crude ether extract was used in

TABLE 2. QUANTITY OF SUDEX SHOOT EXTRACTS PARTITIONED FROM 400 GRAMS DRIED SHOOT MATERIAL AND THEIR INFLUENCE ON RADICLE LENGTH OF CURLY CRESS AT 72 HOURS AT A CONCENTRATION OF 0.33 MG/ML.

Treatment	Quantity extracted (mg)	Radicle length (mm)	Inhibition %
Distilled water control		20.0	
Crude aqueous		12.0	40.0
Hexane	133.6	11.3	43.5
Diethyl ether	394.4	0.6	97.0
Ethyl acetate	286.8	1.3	93.5
Remaining aqueous	88,828.8	17.4	13.0
LSD (0.05)		3.8	

further separations and bioassays in an attempt to isolate sudex inhibitors responsible for the reduction in seedling growth.

Isolation and Characterization of Phytotoxins. Two toxic compounds were isolated from the ether extract of greenhouse-grown sudex material. It was necessary to extract fairly large amounts of dried sudex material (800 g) to obtain enough purified product for bioassay and comparison with synthetic standards. Since the ether extract was a complex mixture, the isolation of both compounds required the use of several chromatographic separation techniques.

After separation using both silica gel and C_{18} flash column chromatography, inhibitory activity, as measured by a cress seed bioassay, was concentrated in one fraction. Following separation via TLC, strong inhibitory activity was associated with zones 2 ($R_f = 0.36$) and 5 ($R_f = 0.58$). Based on similarity of R_f values, compound 1 ($R_f = 0.36$) was tentatively identified as *p*-hydroxybenzoic acid while compound 2 ($R_f = 0.58$) was tentatively identified as *p*-hydroxybenzaldehyde. Both compounds appeared colorless, were strongly visible under UV (254 nm) light, and turned pale yellow after exposure to vanillin and sulfuric acid spray reagent. Identity was confirmed by coelution with known standards on TLC and HPLC.

Dhurrin and *p*-hydroxybenzaldehyde have previously been isolated and identified as major inhibitors of seedling growth in the related species, Johnson grass (*Sorghum halepense* L.) and sorghum (Alsaadawi et al., 1986; Abdul-Wahab and Rice, 1967; Nicollier et al., 1983). Dhurrin is a cyanogenic glycoside that breaks down enzymatically to form *p*-hydroxybenzaldehyde, prussic acid (HCN), and glucose (Conn, 1981). Recently, Nicollier et al. (1983) also found that taxiphyllin, the epimer of dhurrin, was present in Johnson grass rhizomes. Although *p*-hydroxybenzaldehyde and HCN are known inhibitors of

seedling growth, dhurrin and taxiphyllin reduced seedling growth only minimally in tomato and radish bioassays (Nicollier et al., 1983).

Our extraction techniques and bioassays of sudex shoot tissue have shown major biological activity associated only with the presence of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde. It is very likely that *p*-hydroxybenzaldehyde is present as the breakdown product of the cyanogenic glycoside dhurrin. Evidence obtained from UV and NMR spectroscopy suggests that small quantities of dhurrin were also isolated in crystalline form, but possessed relatively little inhibitory activity using the cress bioassay (data not presented). HCN was not detected in the ether extracts of sudex tissue. At room temperatures, *p*-hydroxybenzaldehyde can be converted to *p*-hydroxybenzoic acid. The inhibitory activity of compounds 1 and 2, identified as *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde, were nearly identical to the activity of the synthetic standards used in the cress seed bioassay (data not presented). The synthetic chemicals were utilized to develop dose-response curves for three small-seeded indicator species: cress, lettuce, and radish. The values representing 50% growth reduction (I_{50}) were calculated for each species (Table 3), and with the exception of curly cress, *p*-hydroxybenzoic acid inhibited radicle elongation of seed indicators slightly more than did *p*-hydroxybenzaldehyde. Some difference in sensitivity was also noted among indicators; I_{50} values obtained for lettuce were lower than those of radish and cress, indicating greater sensitivity for lettuce to these compounds. These I_{50} values using the various seed bioassays range from 70 to 140 $\mu\text{g/ml}$ (Table 3). These compounds are inhibitory to radicle elongation in cress, lettuce, and radish at concentrations comparable to other alleged allelochemicals (Rice, 1984). Both compounds are present in large proportions in the ether extracts or sudex shoot tissue.

Quantification of Phytotoxins from Sudex Shoots of Various Ages. Greenhouse-grown sudex residues collected at 7 days of age produced approximately

TABLE 3. INFLUENCE OF *p*-HYDROXYBENZOIC ACID AND *p*-HYDROXYBENZALDEHYDE ON INHIBITION OF RADICLE ELONGATION AT 72 HOURS

Compound	I_{50} value ($\mu\text{g/ml}$) ^a		
	Curly cress	Lettuce	Radish
<i>p</i> -Hydroxybenzoic acid	140.0	70.0	130.0
<i>p</i> -Hydroxybenzaldehyde	113.3	96.7	140.0

^a I_{50} values represent the point at which 50% inhibition of radicle elongation of indicator species occurred. A dose-response curve was created on the basis of mean elongation of 75 seeds at eight concentrations of the chemicals.

four times more dried ether extract per 100 g of dried shoot tissue than did tissue collected at 28 days of age (Table 4). The amounts of the two phyto-inhibitors (*p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde) in tissues of various ages were also compared by HPLC quantification (Table 4). Shoot tissue collected at 7 days of age contained approximately 17 times more *p*-hydroxybenzoic acid and twice as much *p*-hydroxybenzaldehyde than did tissue collected at 28 days of age. In all tissues, *p*-hydroxybenzoic acid was the major component present in the crude ether extracts of sudex tissue (15–59%), with considerably less *p*-hydroxybenzaldehyde present (0.01–0.03%). The figures shown are estimates of the quantities present in the ether extracts and may or may not reflect relative amounts of the two inhibitors present in the whole plant before extraction. However, partitioning with ether did remove the majority of these inhibitors from the aqueous extracts (as observed by TLC and the cress bioassay).

Sudex shoot tissue harvested at 7 days of age (< 10 cm in height) was most inhibitory to seedling growth and also contained the greatest percentage of the two inhibitors. As sudex shoot tissue matured to 28 days of age, both the activity associated with the tissue and the concentration of identified inhibitors decreased dramatically. This is in agreement with the findings of other researchers who note that dhurrin concentration (Conn, 1981) and tissue toxicity (Lehle and Putnam, 1982) are greatest in seedling sorghum. In sudex shoot tissue, the great majority of toxicity appears to be well correlated with the presence of the breakdown products of dhurrin, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. Little direct evidence was previously available to indicate that identified sorghum phytoinhibitors were well correlated with observed inhibition of seedling growth. As Lehle and Putnam (1982) note, this may have resulted from a

TABLE 4. QUANTITY OF ETHER EXTRACT, *p*-HYDROXYBENZOIC ACID AND *p*-HYDROXYBENZALDEHYDE OBTAINED FROM PARTITIONING AND HPLC QUANTIFICATION OF 100 GRAMS OF SUDEX SHOOT RESIDUES OF VARIOUS AGES

Tissue age (days)	Ether extract, mg/100g	Material recovered			
		<i>p</i> -Hydroxybenzoic acid		<i>p</i> -Hydroxybenzaldehyde	
		mg/100 g	%	mg/100g	%
7	230	136.6	59.4	2.6	0.012
14	113	37.1	32.6	2.1	0.019
21	75	13.7	18.3	1.7	0.023
28	55	8.1	14.7	1.6	0.033

lack of standardized methods or suitable bioassays used to quantify inhibitory responses reported.

Identified phenolic compounds may potentially be released from plant tissues by exudation or degradation and may play an important role in the alleged allelopathic activity of both living and dead sudex tissue. Although greatest toxicity was associated with the seedling stage of sudex, the competitive nature, large biomass production, and allelopathic properties exhibited by mature sudex may contribute to its potential role as a weed suppressive summer annual in agroecosystems.

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ROOT-MEDIATED EFFECTS IN CARROT RESISTANCE TO THE CARROT FLY, *Psila rosae*

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Abstract—Field experiments on two different soil types in Ireland assessed the extent and mechanisms of resistance to *Psila rosae* (F.), the carrot fly, with emphasis on the role of the carrot root. Ten carrot cultivars gave consistent results in terms of resistant and susceptible cultivars. Nonpreference oviposition was confirmed as a mechanism, and the use of egg traps, providing differential exposure of the main root, showed this was regulated by root factors, probably chemical constituents. Independent main root resistance to the larva was also confirmed, and this effect was established as consistent with a chemically mediated nonpreference. Antibiosis by the root was demonstrated. Such effects in three different modes indicate that main root properties are crucial in carrot resistance to *Psila* and suggest a pervasive influence of root chemicals on such resistance.

Key Words—Carrot resistance, carrot fly, *Psila rosae*, Diptera, Psilidae, main root factors, nonpreference oviposition, root resistance to larvae, antibiosis, root chemicals.

INTRODUCTION

The first report of resistance in carrot (*Daucus carota* L.) against the carrot fly [*Psila rosae* (F.)] indicated nonpreference in egg-laying as a contributing factor (Åhlberg, 1944). Most subsequent investigations emphasized screening for resistance, which led to the formation in 1976 of a working group of the International Organisation for Biological Control (IOBC) with representatives from nine European countries. Their data from 12 sites demonstrated resistance as having sufficient effect and consistency to suggest a role for resistant cultivars in the integrated control of *Psila* (Ellis and Hardman, 1981); however, the

mechanisms of resistance were not ascertained. These were examined in this laboratory, where we also addressed the role of chemical factors using a range of cultivars different from those of the IOBC (Ryan et al., 1978).

Our field experiments confirmed nonpreference at the level of oviposition and detected a new effect in the form of root resistance to the larva that was independent of resistance to egg-laying (Guerin and Ryan, 1984); that is, two cultivars attracting equally few eggs could sustain widely different degrees of root damage. As such damage is the direct cause of crop losses, it follows that root resistance to the larva is a fundamental requirement in breeding resistance (Guerin and Ryan, 1984). It was not possible to decide if this operated through larval nonpreference or antibiosis, and this question is addressed in the present report. It was also clear that intact roots of resistant varieties released substantially less volatile material than susceptible ones as judged by comparison of steam distillates and of headspace vapors entrapped by Porapak Q (Guerin and Ryan, 1984).

We now report on field experiments with the IOBC range of cultivars. The results confirm nonpreference at the level of oviposition, independent root resistance to the larva, and show, in addition, that the latter is consistent with a chemically mediated nonpreference effect. The results unambiguously demonstrate antibiosis as independently elicited by the root, which also involves chemicals. Finally, and perhaps surprisingly, they indicate that root factors, as distinct from foliage ones, can govern nonpreference oviposition. Collectively, such data establish the root as capable of regulating carrot resistance to *Psila*, probably through its chemical constituents.

METHODS AND MATERIALS

A total of five field experiments were conducted over three years to assess both the extent of resistance to *P. rosae* in carrots and the underlying mechanisms, with special reference to chemical factors. In one we compared only two cultivars using egg-laying traps (details below). In the other four the design was similar to one already reported for use with IOBC cultivars (Ellis and Hardman, 1981). Essentially, there were three blocks each containing one replicate of each cultivar randomized within the block. Block dimensions were 5 × 5 m with 2 m between blocks. All carrot seed was hand-sown in 5-m rows spaced 40 or 50 cm apart within the blocks. Rows were perpendicular to a hedge and began 2 m away from it. Two guard rows terminated the layout of each trial.

Carrot seed of the following eight cultivars tested was supplied by the IOBC through the National Vegetable Research Station (NVRS), Wellesbourne, U.K.: Clause's Original Sytan (hereafter Sytan), Long Chantenay, Vertou L.D. (hereafter Vertou), Danvers Half Long 126, Gelbe Rheinische, Clause's Jaune

Obtuse Du Doubs, St. Valery, Royal Chantenay Elite. They represented a selection of European stocks ranging from susceptible to resistant (Ellis and Hardman, 1981). Caulfield Seed Merchants, Dublin, supplied seed of *Regulus Imperial* and *Chantenay-Red Cored-Elite* representing, respectively, the extremes of resistance and susceptibility previously reported from this laboratory (Guerin and Ryan, 1984).

Extent of Resistance

Two field experiments assessed the extent of resistance on contrasting soil types. All 10 cultivars were compared on peaty soil, comprising layered fen peat with *Menyanthes trifoliata*, non-*Sphagnum* moss and intermittent birch remains over wood fen, at the Peatland Experimental Station, The Agricultural Institute, Lullymore, County Kildare in 1982. The 1983 experiment was made on silty loam soil at University College Dublin, Belfield, using only the eight cultivars as supplied by the IOBC. Both experiments were harvested in late October and the roots graded for damage into five agreed categories of damage (Ellis et al., 1978): 0% (clean), <5%, 5–25%, 26–50%, and >50% of root surface damaged. Numbers and total weight of roots in each grade were recorded for each row. The data were subjected to analysis of variance as: number of roots; percentage unattacked roots; percentage of carrots with less than 5% of root surface damaged, i.e., percentage of marketable roots; and root damage index based on a grading system described by Ellis et al. (1978). These grades ranged from one for marketable roots to four for roots with more than 50% of the surface damaged. To allow for variations between cultivars in root density, the data were subjected to covariance analysis and derived means were compared by the least significant difference (LSD) test.

Mechanisms of Resistance

These were investigated by three experiments in 1981, 1982, and 1983, all on the peatland site of Lullymore. Essentially, the population densities of successive life stages were estimated to compare the mortality of each stage between cultivars.

Egg Sampling. Egg numbers were estimated on nine and 13 occasions for first and second generations of 1982 and on nine occasions for the 1983 experiment, from each of five plants, selected at random, in each row of the center block. Egg densities per cultivar were estimated from those in soil collected in a spoon (5 cm diameter) from the area surrounding the plant within a radius of 5 cm from the root and to a depth of 2.5 cm as previously described (Guerin and Ryan, 1984).

Larval Sampling. Six times during the 1981 experiment and 13 times in 1982, carrots were sampled by taking five random batches of two carrots per

row to give a total of 30 carrot plants for each cultivar (Guerin and Ryan, 1984). In addition to numbers of larvae, observations were made on numbers of attacked roots and mines. A mine is defined as an area of carrot 2 cm in diameter on the surface extending towards the core (Wright and Ashby, 1946).

Pupal Sampling. Pupal numbers were assessed on January 12, 1983, by collecting three soil samples per row from each block, or a total of nine samples per cultivar, using a square metal frame (25 × 25 × 15 cm deep). This was pushed into the soil surrounding the plants with the carrots centered in the frame. Then the soil sample was collected by pulling out the frame with the soil, which was poured into a plastic bag. Carrot roots were counted in the laboratory, and pupae were extracted from the soil by flotation using a saturated solution of magnesium sulfate. Pupae were collected with a paint brush, streaked on white filter paper, and counted. The number of roots per frame was not constant, so pupal numbers are expressed per root rather than per area.

Root Factors

The 1983 experiment differed from the usual design by concentrating on factors affecting egg numbers on only two cultivars, Vertou and Long Chantenay, representing the extremes of resistance and susceptibility, respectively. First, numbers of first-generation eggs were assessed by the spoon method on nine occasions at three-day intervals, from June 22 to August 16, on four plants selected at random from each plot.

Numbers of second-generation eggs were compared by the use of egg traps originally designed for use with cabbage root fly, *Delia radicum (brassicae)* L., (Freuler and Fischer, 1983). They were made of thin layers of felt material rolled down into a disk-shaped trap (5 cm diam. × 2 cm thick; Figure 1). Eggs are laid on the top surface of the traps between the felt layers. We used the traps in two forms: (A) those with a sponge centerpiece that encircled the stem of the plant on the soil surface such that the root was completely covered, and (B) those without such a centerpiece, thus exposing the top of the root. This comparison tested the hypothesis that manipulating the amount of root exposed, and presumably the quantity of root volatiles released, would affect the numbers of eggs laid.

It is generally accepted that larvae feed on the slender side roots before invading the main root (Gorham, 1934; Van't Sant, 1961; Whitcomb, 1938; Beirne, 1971; Städler, 1971; Jones, 1979; Guerin and Ryan, 1984); Overbeck (1978) reported that one third of larval development occurs in such side roots. We sought to compare the extent to which the first-instar larva of *Psila* feeds on side roots of resistant and susceptible cultivars before moving on to the main root. Accordingly, the surfaces of side roots in samples of 40 roots each from Vertou and Long Chantenay, taken at three-day intervals on each of seven occasions from August 4 to 19, 1983, were examined microscopically (×40). In

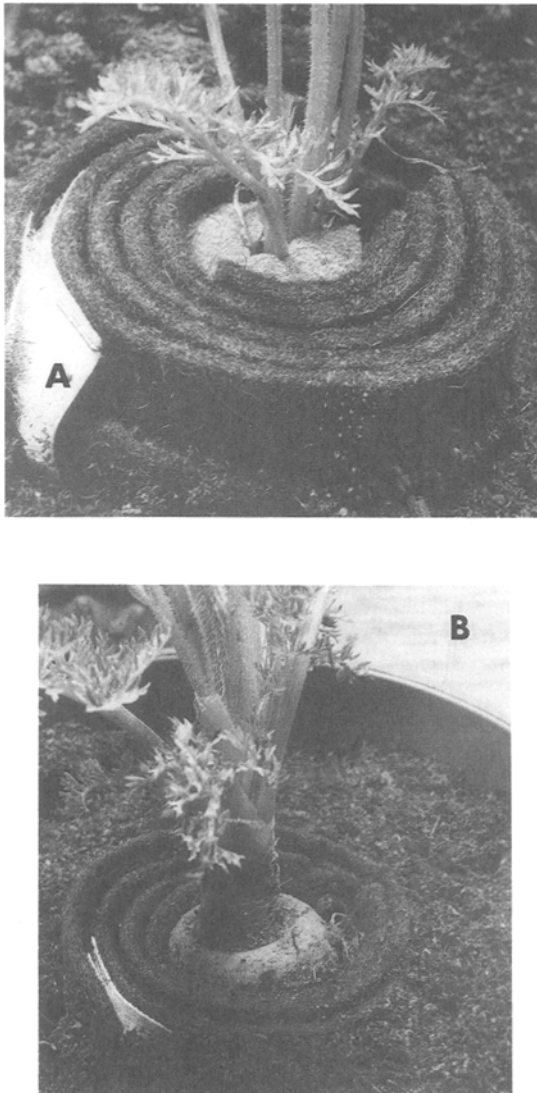


FIG. 1. Egg-traps used to assess the effect on oviposition of exposing the carrot root top. In A The sponge centerpiece completely covers the root top and in B the absence of the centerpiece exposes it. In both, the foliage is unaffected. With the root top covered, flies did not distinguish between Vertou and Long Chantenay in ovipositing, but when it was exposed significantly more eggs were laid on Long Chantenay as in regular field situations. Clearly, the foliage alone is inadequate to elicit this preference and must be supplemented by input from the root which, in the present comparison, is likely to be chemical.

addition, the cotton–blue lactophenol staining method, used to detect nematodes in plants (Souchev, 1970), was exploited in an effort to identify larvae of *P. rosae* in 175 side roots taken from each cultivar.

Statistical Analysis

All data for the experiments on resistance mechanisms relating to numbers of eggs, larvae, and mines were subjected to a square-root transformation ($X = \sqrt{n + 1}$) followed by analysis of variance; means were compared by the Duncan's multiple-range test (Duncan, 1955).

RESULTS

Extent of Resistance

The experiments on peat and mineral soil gave essentially similar results. All differences between cultivars reported below are statistically significant ($P < 0.05$) unless otherwise stated. On peat soil, Vertou had less damage, more unattacked roots, and more marketable roots than Regulus Imperial, St. Valery, and Long Chantenay (Table 1). On mineral soil, Clause's Original Sytan and Vertou had less damage than St. Valery, Danvers Half Long 126, Gelbe Rheinische, Long Chantenay, and Royal Chantenay Elite; more unattacked roots than all the other six cultivars, which did not differ significantly from each other; and more marketable roots than Danvers Half Long 126, St. Valery, Gelbe Rheinische, and Royal Chantenay Elite (Table 2).

We consolidated the results from each experiment into a single ranking system by assigning a value of 1 to the least resistant cultivar under each criterion; values of 10 and 8 were assigned to the most resistant cultivar in peat and mineral experiments, respectively, to take account of the different number of cultivars tested. This allows a possible maximum score of 30 and 24 for each cultivar in the peat and mineral soil experiments, respectively. For comparisons between experiments, cultivars were then rated by expressing their scores as a percentage of the maximum possible score (Tables 1 and 2). On peat soil, Vertou was the most resistant cultivar (100%), followed by Gelbe Rheinische (87%), and Regulus was the most susceptible (10%). On mineral soil, the two most resistant cultivars were Sytan (100%) and Vertou (88%), and Danvers Half Long 126 (17%) was the most susceptible.

Mechanisms of Resistance

In the 1981 experiment, when observations were confined to the second generation, more larvae and more mines per root were recorded on Long Chantenay than on all the other cultivars, which did not differ significantly from each other in either respect (Table 3).

TABLE 1. RANKED ORDER UNDER THREE CRITERIA OF CARROT FLY DAMAGE ON TEN CARROT CULTIVARS AT LULLYMORE (PEAT SOIL) IN 1982 WITH CONSOLIDATION INTO SINGLE RANKING TO EXPRESS EXTENT OF RESISTANCE^a

Cultivar	Root damage index ^b	Cultivar	Clean roots (%) ^c	Cultivar	Marketable roots (%) ^d	Cultivar	Score as % of max. possible ^e
Vertou	1.59 a	Vertou	48.5 a	Vertou	63.9 a	Vertou	100
Royal Chantenay Elite	1.68 ab	Gelbe Rheinische	44.5 ab	Gelbe Rheinische	63.9 ab	Gelbe Rheinische	87
Gelbe Rheinische	1.75 ab	Royal Chantenay Elite	39.0 abc	Royal Chantenay Elite	57.5 ab	Royal Chantenay Elite	83
Sytan	1.87 abc	Clause's Jaune Obtuse	38.2 abc	Clause's Jaune Obtuse	51.3 abc	Clause's Jaune Obtuse	63
		Du Doubts		Du Doubts		Du Doubts	
Chantenay-Red	1.91 abc	Chantenay-Red	38.0 abc	Sytan	49.6 abc	Sytan	60
Cored-Elite		Cored-Elite					
Clause's Jaune Obtuse	1.96 abc	Sytan	36.7 abc	Chantenay-Red	49.5 abc	Chantenay-Red	57
Du Doubts				Cored-Elite		Cored-Elite	
Danvers Half Long	1.99 abc	Danvers Half Long	35.8 abc	Danvers Half Long	48.3 abc	Danvers Half Long	40
		126		126		126	
St. Valery	2.08 bcd	Long Chantenay	31.0 bcd	Long Chantenay	43.8 bcd	Long Chantenay	27
Long Chantenay	2.08 bcd	St. Valery	29.4 bcd	St. Valery	42.3 bcd	St. Valery	23
Regulus Imperial	2.22 cd	Regulus Imperial	23.8 cd	Regulus Imperial	36.3 cd	Regulus Imperial	10

^a Means in the same column with different letters are significantly different ($P < 0.05$).

^b Mean damage scores which range from 1 for marketable roots to 4 for root with more than 50% of the surface damaged.

^c Unattacked roots.

^d Percentage of carrots with less than 5% of root surface damaged.

^e Scoring system based by assigning 1 to the least resistant and 10 to the most resistant cultivars with corresponding intervening values.

TABLE 2. RANKED ORDER UNDER THREE CRITERIA OF CARROT FLY DAMAGE ON EIGHT CARROT CULTIVARS AT BELFIELD (MINERAL SOIL) IN 1983 WITH CONSOLIDATION INTO SINGLE RANKING TO EXPRESS THE EXTENT OF RESISTANCE.^a

Cultivar	Root damage index ^b	Cultivar	Clean roots (%) ^c	Cultivar	Marketable roots (%) ^d	Cultivar	Score as % of max. possible ^e
Sytan	1.74 a	Sytan	46.5 a	Sytan	61.1 a	Sytan	100
Vertou	2.07 ab	Vertou	38.6 a	Vertou	50.2 ab	Vertou	88
Clause's Jaune Obtuse	2.67 bc	Long Chantenay	18.5 b	Clause's Jaune Obtuse	29.6 bc	Clause's Jaune Obtuse	67
Du Doubs				Du Doubs		Du Doubs	
Royal Chantenay Elite	2.75 c	Royal Chantenay Elite	18.2 b	Long Chantenay	29.4 bc	Long Chantenay	63
Long Chantenay	2.75 c	Clause's Jaune Obtuse	16.9 b	Royal Chantenay Elite	26.8 c	Royal Chantenay Elite	58
		Du Doubs					
Gelbe Rheinische	2.83 c	Gelbe Rheinische	15.6 b	Gelbe Rheinische	23.6 c	Gelbe Rheinische	38
Danvers Half Long 126	2.89 c	St. Valery	12.6 b	St. Valery	22.3 c	St. Valery	21
St. Valery	2.91 c	Danvers Half Long 126	6.4 b	Danvers Half Long 126	18.9 c	Danvers Half Long 126	17

^aMeans in the same column with different letters are significantly different ($P < 0.05$).

^bMean damage scores, which range from 1 for marketable roots to 4 for root with more than 50% of the surface damaged.

^cUnattacked roots.

^dPercentage of carrots with less than 5% of root surface damaged.

^eScoring system based by assigning 1 to the least resistant and 8 to the most resistant cultivars with corresponding intervening values.

TABLE 3. RANKED ORDER IN TERMS OF MEAN NUMBERS OF LARVAE AND MINES FOR TEN CARROT CULTIVARS AT LULLYMORE (PEAT SOIL) IN 1981 TO ASSESS MECHANISMS OF RESISTANCE^a

Cultivar	No. of larvae ^b	Cultivar	No. of mines ^b	Cultivar	No. of mines/invaded larva
Vertou	1.05 a	Vertou	1.07 a	Sytan	1.00
Gelbe Rheinische	1.06 a	Gelbe Rheinische	1.8 a	Long Chantenay	1.00
Chantenay-Red	1.07 a	Chantenay-Red	1.08 a	Regulus Imperial	1.01
Cored-Elite		Cored-Elite		Chantenay-Red	1.01
St. Valery	1.08 a	Sytan	1.09 a	Cored-Elite	
Danvers Half Long	1.08 a	St. Valery	1.11 ab	Vertou	1.02
Sytan	1.09 a	Danvers Half Long	1.11 ab	Gelbe Rheinische	1.02
Royal Chantenay Elite	1.10 a	126		Clause's Jaune Obtuse	1.02
Clause's Jaune Obtuse	1.11 a	Regulus Imperial	1.12 ab	Du Doubs	
Du Doubs	1.11 a	Royal Chantenay Elite	1.13 ab	St. Valery	1.03
Regulus Imperial	1.11 a	Clause's Jaune Obtuse	1.13 ab	Royal Chantenay Elite	1.03
Long Chantenay	1.19 b	Du Doubs		Danvers Half Long	1.03
		Long Chantenay	1.19 b	126	

^a Means in the same column with different letters are significantly different ($P < 0.05$).

^b Data transformed $\sqrt{n + 1}$.

In the 1982 experiment (first generation), fewer eggs were laid on Vertou, Sytan, and three other cultivars than on Royal Chantenay Elite, Regulus Imperial, and Long Chantenay (Table 4). There was, however, no significant difference between the numbers of larvae subsequently recorded on the roots of these cultivars (Table 4). Vertou, Clause's Jaune Obtuse Du Doubs, and Sytan had fewer mines per root than Long Chantenay, Chantenay-Red Cored-Elite, and Royal Chantenay Elite. The ratio of eggs laid to larvae established in main roots ranged from 7 (Gelbe Rheinische) to 11 (Long Chantenay) (Table 4).

Using the scoring system, Vertou was the most resistant (100%) followed by Clause's Jaune Obtuse Du Doubs (90%), St. Valery (77%), and Sytan (73%). In contrast, Long Chantenay was the most susceptible (27%).

Experimental results from the second generation were consistent with those from the first. Vertou had fewer eggs than Danvers Half Long 126 and Gelbe Rheinische. Furthermore, Vertou had fewer larvae than Gelbe Rheinische, Long Chantenay, Danvers Half Long 126, and Regulus Imperial. The ratio of eggs laid to larvae established in main roots ranged from 10 (Danvers half Long 126) to 12 (Sytan and Vertou) (Table 5). Vertou, Sytan, and three other cultivars had fewer mines per root than Gelbe Rheinische, Long Chantenay, Danvers Half Long 126, and Regulus Imperial (Table 5). Larval damage increased on all cultivars from the middle of September and peaked in November. A comparison between Vertou (resistant) and Long Chantenay (susceptible) showed that consistently more eggs, larvae, mines, and damaged roots were recorded on Long Chantenay than on Vertou. Percentage larval survival to the pupal stage ranged from 66% (St. Valery) to 98% (Vertou). The same scoring system showed Vertou again the most resistant cultivar (82%), followed by Clause's Jaune Obtuse Du Doubs (80%) and Sytan (78%). Long Chantenay was the third most susceptible cultivar (38%).

In the 1983 experiment (first generation) fewer ($P < 0.001$) eggs were laid on Vertou than on Long Chantenay as judged by the flotation method (Table 6). In the second generation, the use of egg trap A (root top enclosed) abolished this difference. But use of egg trap B (root top exposed) restored the significant difference between Vertou and Long Chantenay (Table 6). This unambiguously demonstrates that the root can regulate preference/nonpreference oviposition by the female fly.

The mean number of side roots per carrot root of the two cultivars Vertou and Long Chantenay was identical. Lactophenol staining did not detect *Psila* larvae inside such side roots. However, larval feeding areas were readily identified by examination with a binocular microscope. Each comprised a shallow pit in the epidermis (<2 mm long and 50–100 μ m deep), with a rust-colored perimeter. Alternatively this took the form of a narrow scar, more than 2 mm long. However, there was no significant difference between mean numbers of side roots infested on Vertou (1.3) and Long Chantenay (1.2) ($N = 7$).

TABLE 4. RANKED ORDER IN TERMS OF MEAN NUMBERS OF EGGS, LARVAE AND MINES PER ROOT FOR TEN CARROT CULTIVARS AT LULLYMORE (PEAT SOIL) DURING FIRST GENERATION 1982 WITH CONSOLIDATION INTO SINGLE RANKING TO ASSESS MECHANISMS OF RESISTANCE^a

Cultivar	No. of eggs ^b	Cultivar	No. of larvae ^b	Cultivar	No. of mines ^b	Cultivar	Score as % of max. possible ^c
Vertou	1.12 a	Vertou	1.09 a	Vertou	1.36 a	Vertou	100
Clause's Jaune Obtuse	1.16 a	Clause's Jaune Obtuse	1.12 a	Clause's Jaune Obtuse	1.41 ab	Clause's Jaune Obtuse	90
Du Doubs		Du Doubs		Du Doubs		Du Doubs	
St. Valery	1.17 a	St. Valery	1.14 a	Sytan	1.42 ab	St. Valery	77
Sytan	1.22 a	Sytan	1.15 a	St. Valery	1.53 abc	Sytan	73
Chantenay-Red	1.27 a	Long Chantenay	1.15 a	Regulus Imperial	1.56 abc	Danvers Half Long	43
Cored-Elite						126	
Gelbe Rheinische	1.29 ab	Danvers Half Long	1.17 a	Gelbe Rheinische	1.65 cd	Gelbe Rheinische	37
		126					
Danvers Half Long	1.40 ab	Royal Chantenay Elite	1.19 a	Danvers Half Long	1.68 cd	Regulus Imperial	37
		126		126			
Royal Chantenay Elite	1.55 b	Regulus Imperial	1.19 a	Royal Chantenay Elite	1.78 d	Royal Chantenay Elite	33
Regulus Imperial	1.57 b	Chantenay-Red	1.21 a	Chantenay-Red	1.78 d	Chantenay-Red	33
		Cored-Elite		Cored-Elite		Cored-Elite	
Long Chantenay	1.57 b	Gelbe Rheinische	1.22 a	Long Chantenay	1.83 d	Long Chantenay	27

^aMeans in the same column with different letters are significantly different ($P < 0.05$).

^bData transformed $\sqrt{n} + 1$.

^cScoring system based by assigning 1 to the least resistant and 10 to the most resistant cultivars with corresponding intervening values.

TABLE 5. RANKED ORDER FOR MEAN NUMBERS OF EGGS, LARVAE AND MINES PER ROOT AND PERCENTAGE LARVAL SURVIVAL TO PUPAL STAGE AT LULLYMORE (PEAT SOIL) DURING SECOND GENERATION 1982 WITH CONSOLIDATION INTO SINGLE RANKING^a

Cultivar	No. of eggs ^b	Cultivar	No. of larvae ^b	Cultivar	No. of mines ^b	Cultivar	Survival (% larvae to pupae)	Cultivar	Score as % of max. possible ^c
Vertou	1.29 a	Vertou	1.31 a	Vertou	1.34 a	St. Valery	65.9	Vertou	82
Royal Chantenay Elite	1.40 ab	Sytan	1.39 ab	Sytan	1.43 ab	Gelbe Rheinische	84.3	Clause's Jaune Obtuse Du Doubs	80
Clause's Jaune Obtuse Du Doubs	1.41 ab	Clause's Jaune Obtuse Du Doubs	1.40 ab	Clause's Jaune Obtuse Du Doubs	1.45 ab	Long Chantenay	87.9	Sytan	78
Sytan	1.43 ab	St. Valery	1.41 ab	Chantenay-Red Cored-Elite	1.45 ab	Clause's Jaune Obtuse Du Doubs	88.3	St. Valery	66
Chantenay-Red Cored-Elite	1.56 ab	Chantenay-Red Cored-Elite	1.41 ab	St. Valery	1.45 ab	Sytan	91.4	Chantenay-Red Cored-Elite	56
St. Valery	1.57 ab	Royal Chantenay Elite	1.46 ab	Royal Chantenay Elite	1.49 abc	Royal Chantenay Elite	94.2	Royal Chantenay Elite	54
Long Chantenay	1.59 ab	Gelbe Rheinische	1.57 bc	Gelbe Rheinische	1.59 bcd	Danvers Half Long 126	96.7	Gelbe Rheinische	48
Regulus Imperial	1.61 ab	Long Chantenay	1.58 bc	Long Chantenay	1.63 bcd	Regulus Imperial	97.6	Long Chantenay	38
Danvers Half Long 126	1.64 b	Danvers Half Long 126	1.64 bc	Danvers Half Long 126	1.66 cd	Chantenay-Red Cored-Elite	97.6	Danver Half Long 126	28
Gelbe Rheinische	1.72 b	Regulus Imperial	1.71 c	Regulus Imperial	1.73 d	Vertou	97.9	Regulus Imperial	20

^aMeans in the same column with different letters are significantly different ($P < 0.05$).

^bData transformed $\sqrt{n + 1}$.

^cScoring system based by assigning 1 to the least resistant and 10 to the most resistant cultivars with corresponding intervening values.

TABLE 6. COMPARISON OF VERTOUS (RESISTANT) AND LONG CHANTENAY (SUSCEPTIBLE) IN EGG NUMBERS USING SOIL SAMPLING DURING FIRST GENERATION AND EGG TRAPS DURING SECOND GENERATION AT LULLYMORE (PEAT SOIL) IN 1983

Carrot cultivar	First generation	Second generation	
	No. of eggs/root (soil sample)	No. of eggs per trap A ^a	No. of eggs per trap B ^b
Vertou	1.88	1.05	1.03
Long Chantenay	2.54	1.07	1.41
Significance	$P < 0.001$	NS ^c	$P < 0.05$

^aTrap A was with a sponge centerpiece.

^bTrap B was without centerpiece.

^cNot significant.

DISCUSSION

Vertou was consistently resistant: it was the most resistant cultivar on peat and the second most resistant on mineral soil. Sytan's ranking improved from sixth most resistant on peat to first place on mineral soil. At Wellesbourne, U.K., more sinuous mines but fewer nibbles were observed on carrots from fen or peaty soil than on those from mineral soil, suggesting that larvae mined more readily in tissues of peat-grown carrots (Ellis et al., 1978). Such a mechanism could contribute to Sytan's elevation to first place on mineral soil in our experiments. Similarly, Long Chantenay was the second most resistant cultivar in 1982 (peat soil) but the fourth resistant cultivar in 1983 (mineral). Overall, our data are quite consistent with the results of similar experiments in five European countries (Ellis and Hardman, 1981). Using the single criterion of percentage undamaged roots, they also rated Sytan and Vertou as the two most resistant cultivars. Their most susceptible cultivar was St. Valery, with Danvers in second place. Excluding *Regulus Imperial*, which was not considered by them, we found the most susceptible cultivars to be St. Valery and Long Chantenay in 1982 and Danvers and St. Valery in 1983. Thus, we confirm Sytan and Vertou as the most resistant cultivars and St. Valery as among the most susceptible.

In regard to mechanisms, highly significant differences in egg numbers were exhibited by different cultivars during the first ($P < 0.001$) and second ($P < 0.01$) generations of 1982. Resistant Vertou had 29% fewer eggs than susceptible Long Chantenay in the first generation of 1982. Such a response confirms nonpreference by the female *Psila* (Åhlberg, 1944; Guerin and Ryan, 1984). The experiment with egg-laying traps showed that exposing only the foliage was insufficient to reproduce differential egg numbers found between

Vertou and Long Chantenay; it was essential to also expose the respective root tops. This effect may be assignable to root volatiles, i.e., attractants and/or oviposition stimulants in root scent.

Significantly fewer eggs were laid on foliage of resistant Sytan than on that of susceptible Danvers in laboratory experiments (Guerin and Städler, 1982), which is consistent with the data from our field experiments. However, this preference was abolished when artificial leaves impregnated with leaf surface extracts from the two cultivars were compared in the laboratory. Furthermore, in a comparison of the effect of headspace vapor, Sytan attracted significantly more eggs than Danvers. Hence, the effect of contact with foliage was reproduced by neither extracts nor headspace volatiles of foliage. It was acknowledged that the observations took no account of root odor (Guerin and Städler, 1982). The present data indicate that input from the root contributes significantly to differential egg laying.

The second mechanism confirmed by our field data was resistance by the main root to larval attack as judged by numbers of larvae, mines, pupae, and damaged roots. This is exemplified by a comparison of Vertou with Long Chantenay and *Regulus Imperial* in the second generation of 1982. Cultivars Long Chantenay and *Regulus Imperial* had similar egg numbers to Vertou but sustained significantly more larvae, damaged roots, and mines (Table 5). Accordingly, these root effects reflect not only egg numbers but also indicate direct root resistance against the larva. This effect was first reported from a different set of cultivars by Guerin and Ryan (1984).

Present data indicate that the number of side roots would not affect root resistance in the two extreme cultivars (Vertou and Long Chantenay) as the mean number of side roots did not differ significantly between the two cultivars. Furthermore, detailed examination of the anatomy of the side roots gave no evidence of differential feeding or differential invasion. Thus, by elimination, root resistance to larval invasion must operate through the main root.

There was no evidence of more dead or stunted larvae immediately after establishment in any cultivar, eliminating antibiosis at that time as a mechanism. This suggests that decreased invasion, i.e., nonpreference exerted by the main root, governs larval establishment in such roots. At least five compounds, α -ionone, β -ionone, bornyl acetate, biphenyl, and 2,4-dimethyl styrene, attract larvae to roots (Ryan and Guerin, 1982). One compound, *trans*-2-nonenal, repelled and killed the larvae in laboratory experiments (Guerin and Ryan, 1980). Furthermore, combined concentrations of the attractants, but especially of 2,4-dimethyl styrene, were fivefold greater in the main root of the susceptible Chantenay-Red Cored-Elite as compared with resistant *Regulus Imperial* (Guerin and Ryan, 1984). Accordingly, it is reasonable to conclude that nonpreference, as influenced by chemical cues, affects root resistance to the larva.

Another possible resistance mechanism by the main root is antibiosis

against the feeding larva, i.e., if after prolonged feeding, larval growth, development, and survival were decreased (Campbell, 1983). In the second generation of 1982, larval survival in St. Valery and Sytan was 32% and 7% less, respectively, than in Vertou (100% survival). So antibiosis is another mechanism by which main root resistance operates, although it does not materially contribute to the resistance of Vertou and Sytan.

Antibiosis has previously been assigned to carrot on the basis of differential invasion of roots from inoculation with equal numbers of eggs (Guerin et al., 1981). However, the result did not preclude differential invasion due to non-preference behavior by the larvae in response to root chemicals. The present data provide an unambiguous demonstration of antibiosis. As this mechanism takes effect through physiological inhibitors, toxins, and decreased nutrient levels (Beck, 1965), it is reasonable to implicate root chemicals in its action against the *Psila* larva.

In summary, the present data identify root-mediated resistance as operating by: (1) eliciting fewer eggs (nonpreference by the female), (2) independently decreasing larval invasion (nonpreference by the larvae), and (3) antibiosis against the root-established larvae. Accordingly, root-mediated resistance has a pervasive role in carrot resistance to *Psila*. Root chemicals are already established as contributing to the second of these mechanisms (Guerin and Ryan, 1984), but it is now apparent that they also contribute to both the former and the latter. Accordingly, main root chemical constituents may be crucial in carrot resistance to *Psila*.

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ISOLATION, IDENTIFICATION, AND BIOASSAY OF CHEMICALS AFFECTING NONPREFERENCE CARROT-ROOT RESISTANCE TO CARROT-FLY LARVA

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Abstract—Roots of the carrot cultivars Vertou L.D. (resistant) and Long Chantenay (susceptible) were subjected to detailed chemical analysis to identify extracts and compounds influencing larval host-finding (preference/non-preference) behavior and to compare concentrations of these compounds in resistant and susceptible cultivars. Vertou yielded threefold less volatile material in headspace extracts of pureed roots. Extracts of chopped root in methanol, steam, hexane, and chloroform were inactive in behavioral assays. However, ether extracts were active and their hydrocarbon and carbonyl-rich fractions contained potent attractants. The principal constituent of the carbonyl-rich fraction of each cultivar was the carotatoxin complex comprising the neurotoxin falcarinol (carotatoxin), falcarindiol, and falcarindiol monoacetate, the latter compound here reported for the first time from carrot. Falcarinol (50 and 100 μg) was active in a behavioral assay, and all three ingredients of the complex were potent electrophysiological stimuli, eliciting maximum single unit responses to source concentrations of 10 ng. Furthermore, the complex was more abundant by about 1000 $\mu\text{g}/\text{root}$ in Long Chantenay. As this comprised extra amounts of 70, 862, and 110 μg of falcarinol, falcarindiol, and falcarindiol monoacetate, respectively, the observed differences seem both behaviorally and physiologically relevant. It is generally accepted that coevolution has transformed the role of many toxins into host-location cues, but this seems a relatively rare example of a neurotoxin (falcarinol) evincing, in decreased concentrations, nonpreference host resistance.

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This evolved response to a toxin present in large concentrations is contrasted with that to *trans*-2-nonenal, which paralyzes and kills the larva and is present in only trace amounts in the root.

Key Words—Carrot *Daucus carota*, *Psila rosae*, Diptera, Psilidae, carrot-fly larva, root chemicals, carbonyl-rich fraction, falcarinol, falcarindiol, falcarindiol monoacetate, electrophysiology, neurotoxin, carrot resistance factor.

INTRODUCTION

The carrot root influences three principal mechanisms of carrot resistance to carrot fly, namely, nonpreference oviposition, independent nonpreference by the invading larva, and antibiosis against the feeding larva (Guerin and Ryan, 1984; Maki and Ryan, 1989). Of these, decreased oviposition is inadequate for plant protection, as cultivars with significantly fewer eggs nevertheless sustained significantly more root damage (Guerin and Ryan, 1984; Maki and Ryan, 1989). Thus, root resistance to the larva is critical for successful carrot resistance.

There is an association between increased carrot resistance and decreased release of root volatiles, as indicated both by solvent extraction and headspace entrapment. Specifically, the following five compounds significantly preferred by the larva were released in smaller concentrations by roots of resistant cultivars: bornyl acetate; biphenyl, α -ionone, β -ionone, and 2,4-dimethylstyrene (Guerin and Ryan, 1984).

This report further investigates the relationship between root chemistry and root resistance to the larva by examining larval responses to a variety of root extracts, fractions, and individual compounds using both behavioral and electrophysiological assays.

METHODS AND MATERIALS

Extraction and Quantification of Root Volatiles. Headspace extracts were derived from roots of Vertou and Long Chantenay by separate entrapment of their volatiles on the absorbents Porapak Q and Tenax GC (60–80 mesh). Weighed batches of each cultivar's roots, pureed in a food blender with periodic addition of ice, were placed in foil-covered glass tubes 62 cm long \times 9 cm diam. and simultaneously extracted. Air, purified over activated charcoal, diverged to pass over each puree, then through two flow meters set at 0.1 liter/min, and finally through two foil-covered tubes each containing 5 g absorbent. After 48 hr, the purees were discarded and replaced by fresh batches in the washed tubes. Thus ca. 1 kg of each cultivar was extracted for 70–80 hr. Pora-

pak was activated as previously described (Ryan and Guerin, 1982) and Tenax GC was activated by heating at 275°C for 5 hr in a nitrogen stream at 0.1 liter/min. Volatiles from each source were separately extracted in a Soxhlet apparatus for 24 hr in diethyl ether and for a further 24 hr in methanol, solvents of choice for nonpolar and polar compounds, respectively. After concentration by rotary evaporation and exposure to a nitrogen stream, such extracts were quantitatively examined by gas-liquid chromatography (details in Table 1).

The following extractions of chopped root were also made: steam-distillation, separation in water-hexane and water-chloroform mixtures, extraction in methanol followed by fractionation (Figure 1), and extraction in ether; only the latter yielded consistently active fractions (Figure 2). The active carbonyl-rich fractions from the ether extracts were further subdivided by repeated column chromatography on silica gel. The eluants were *n*-hexane-diethyl ether in the successive ratios 19:1, 4:1, 2:1, and 1:1. Eluant samples were monitored by TLC or GLC and isolates were identified by GC-MS (VG Analytical 7070E mass spectrometer, PYE 204 gas chromatograph; Finnigan-Mat INCOS 2400 data system) and [¹H]NMR (Perkin Elmer 12 RB), supplemented by high-field [¹H]NMR (Jeol GX 270). (±)-2-Methoxy-3-*sec*-butylpyrazine was an individual compound of particular interest as it has been claimed to produce the distinctive odor of carrot (Cronin and Stanton, 1976). It was not commercially available and has been recorded in concentrations too small for easy extraction (<1 ppm) (Cronin and Stanton, 1976). Accordingly, it was synthesised as a racemic mixture (F. Abe, In preparation).

TABLE 1. YIELD OF VOLATILES EXTRACTED WITH VARIOUS ADSORBENTS AND SOLVENTS FROM ROOTS OF CARROT CULTIVARS VERTOU (V, RESISTANT) AND LONG CHANTENAY (LC, SUSCEPTIBLE)^b

Adsorbent	Solvent	Volatile yield (μg/80 g pureed root tissue/24 hr) of cultivars ^a		Ratio of V to LC
		Vertou	Long Chantenay	
Porapak Q	Diethyl Ether	539	1410	1 : 2.6
	Methanol	18	21	1 : 1.4
Tenax G C	Diethyl Ether	1251	4019	1 : 3.2
	Methanol	273	503	1 : 1.8

^a80 g represents the mean weight of a mature root for carrots sown in June 1984 and harvested in March 1985. Values represent means of two replicates differing by less than 10%.

^bTypical operating conditions: Hewlett-Packard 5890 GC linked to automatic computing integrator 3380 Å; column, glass 10 m long, 530 μm diameter, coated with Carbowax 20 M, which gives superior resolution of carrot volatiles (Ryan and Guerin, 1982); injection port temperature 220°C, detector temperature 220°C; oven temperature 100°C for 1 min followed by a programmed rise of 10°C/min for 10 min to 170°C.

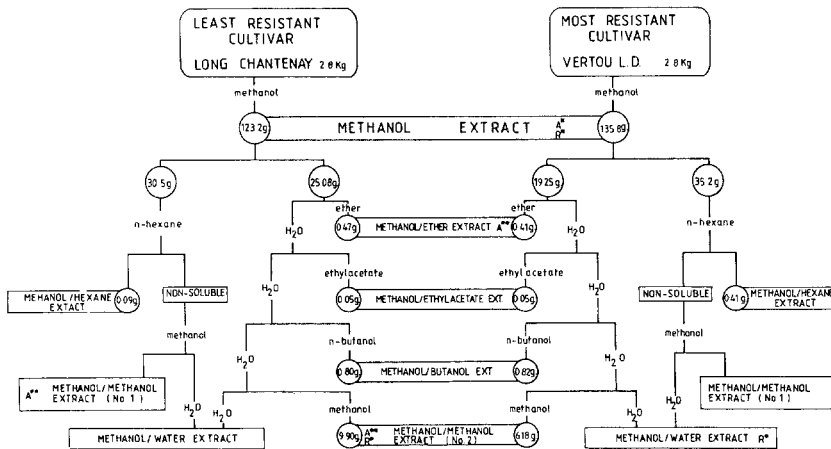


FIG. 1. Yields, effects, and potencies of fractions derived from methanol extracts of Long Chantenay and Vertou carrot roots. Typically, fractions were bioassayed by the box method in concentrations of 0.01, 0.5, and 1.0 mg/disk with some additional observations at 5.0 mg/disk. Encircled values are yields. Effects and potencies: A, attractive, R, repellent; * $P < 0.05$; ** $P < 0.01$. Corresponding values of $R(\%)$, derived from $(T - C/T + C) \times 100$ and rounded to the nearest integer (details in text), were as follows: methanol extract, Vertou, $R = 17\%$ at 0.1 mg/disk and -20% at 0.5 mg/disk; methanol-ether-extract, Vertou, $R = 38\%$ at 1.0 mg/disk; methanol-methanol extract No. 1, Long Chantenay $R = 29\%$ at 0.5 mg/disk; methanol-methanol extract No. 2 Long Chantenay, $R = 28\%$ at 1.0 mg/disk and -20% at 5.0 mg/disk; methanol-water extract, Vertou, $R = -26\%$ at 1.0 mg/disk.

Behavioral Assays. Root extracts were subjected to one of two behavioral assays. The first was based on the method used to observe larval responses of the onion fly, *Delia antiqua* (Meigan), to sulfur-bearing chemicals (Soni and Finch, 1979). It employed a Petri dish (10 cm diam.) lined with a 9-cm, damp, black filter paper (Schleicher and Schull No. 551), marked with a pencil into quarters designated test, control, upper neutral, and lower neutral, respectively. Typically, test chemicals were introduced by adding 100 μ l of stimulus solution onto an elderberry pith disk (1.5 cm diam. \times 0.3 mm thick, preextracted with solvent) (test) in concentrations of 0.05, 0.1, or 1 mg/100 μ l; another pith disk treated with 100 μ l solvent (control) was placed in the opposite sector. After evaporation of solvent, 20–30 third-instar larvae, deprived of food for 24 hr, were placed in the center of each test chamber using a soft paint brush. Five replicates (chambers) were used for each concentration. Test chambers containing larvae were kept in the dark for 15 min at $20 \pm 1^\circ\text{C}$, after which the number of larvae in each sector was counted and recorded.

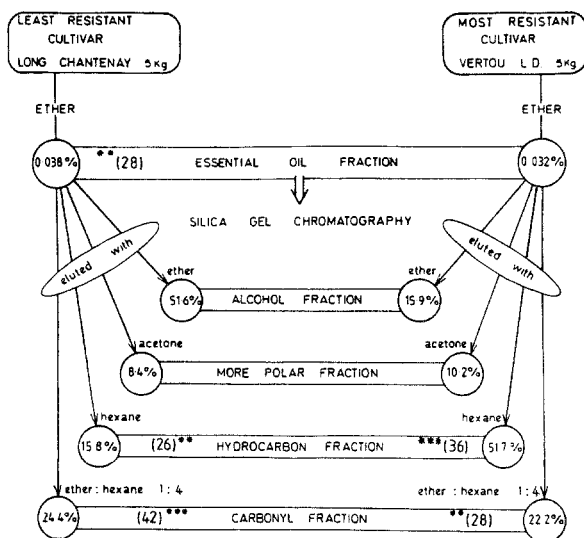


FIG. 2. Yield and potencies of fractions derived from ether extracts of Long Chantenay and Vertou carrot roots. Each batch of roots was extracted with ether and the solvent evaporated in vacuo at 40°C. Each ether extract was loaded on to a column of silica gel (Kiesel Gel 60, Merck, 20 times the extract weight) and eluted with the solvents indicated. Fractions at concentrations of 0.5, 1.0, 2.0, and 4.0 mg/disk were bioassayed by the box method (details in Methods and Materials). All significant responses were elicited at 1.0 mg/disk; ***P* < 0.01; ****P* < 0.001; values in parentheses are *R*(%), see text for details. The yields of ether-extracted oil are equivalent to 380 and 320 ppm for Long Chantenay and Vertou, respectively. On the basis of a mean root weight of 80 g, this is equivalent to an extra 4.80 mg oil from a Long Chantenay root.

The second method employed a lidded rectangular Perspex chamber (15 × 4 × 4 cm deep). The floor was lined with damp filter paper (Whatman No. 40) marked with pencil at the midline to give test and control halves. As before, a pith disk treated with the test chemical (usually 0.001, 0.01, 0.05, 0.1, and 0.5 mg/disk) was placed at the far end of the test half, a corresponding disk treated with solvent placed in the other end of the control half, and 20–30 unfed larvae were employed per replicate. Five lidded chambers were kept in the dark for 15 min, after which time the number of larvae in each half was recorded. Both methods gave essentially similar results when tested with the same compounds.

All data were subjected, first, to a χ^2 test for homogeneity and homogenous data were then further assessed by the *t* test. It was convenient to score larval responses in particular experiments according to the formula: $R(\%) = (T - C /$

$T + C) \times 100$, where T is the number of larvae on the test side of the chamber, and C is the number of larvae on the control side of the chamber.

Electrophysiological Assays. The experimental set-up was housed in an earthed Faraday cage into which ran a stimulus delivery system (Figure 3). Air flow was directed through three solenoids controlled by a timer switch opening one valve for 1 sec every 5 min; it could also be operated manually. The valve directed air over the stimulus chemical applied as 20 μ l to a folded piece of filter paper (12 cm) contained in a glass cartridge (54 mm long and 10 mm diameter) with ground glass end. The other two lines terminated in a control (solvent) cartridge and an empty cartridge, respectively, the latter providing a continuous flow of purified air to remove stimulus molecules from the vicinity of the preparation.

The principal electronic control unit was the WPI Micro-probe system model M-707 equipped with a probe, which was gold-plated, epoxy-sealed, and contained the first stage of amplification ($\times 1$). Plugged into this was a micro-electrode holder having a molded Ag-AgCl half cell into which the recording microelectrode was inserted through a rubber gasket. This microprobe system had the following features: current passing and low noise, drift-free performance, bandwidth limiting filter, notch filter, selectable bridge balance range, and a probe test. The system was connected to both a Phillips PM 8010 double pen recorder and to a Telequipment DM 64 storage oscilloscope. Recording and reference microelectrodes had tip diameters of 0.5 and 5.0 μ m, respec-

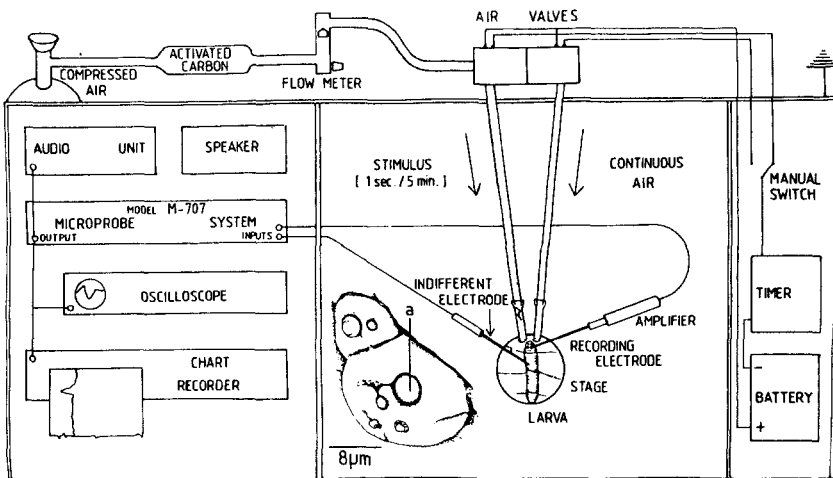


FIG. 3. A block diagram of the electrophysiological set-up. Inset: diagrammatic representation of the larval group B sensilla indicating the ampullaceous sensillum (a), from which recordings were made.

tively, and were loaded with Ringer solution identical in the following constituents to that of receptor lymph from *Antheraea polyphemus* (K.E. Kaissling, personal communication: glucose (22.50 mM/liter), KCl (171.90), KH_2PO_4 (9.17), K_2HPO_4 (10.83), MgCl_2 (3.00), CaCl_2 (1.00), NaCl (25.00), and HCl (1.50); pH 6.5 and 475 mosm).

Third-instar larvae, unfed for 4 hr at room temperature, were immobilized on a disk-shaped rubber stage (3 cm diam.) by knots of human hair. The larva bears on its cephalic lobes 24 sensilla arranged in three groups (A, B, and C), of which group B comprises six evident sensilla on each side of the ventral midline: one ampullaceous (3 μm diam.) and three basiconic on a cuticular projection; dorsolateral to this are two additional sensilla, one styloconic and one basiconic (Ryan and Behan, 1973) (see inset to Figure 3). This group of sensilla seems analogous to the terminal organ of the house fly larva and the anterior organ of the larvae of the onion and seed corn flies (Yamada et al., 1981; Honda and Ishikawa, 1987a). The latter organ has been associated, through electrophysiological recordings, with a gustatory function, but, because the capillary tip touched the whole organ, it was not possible to distinguish the functions of individual sensilla (Honda and Ishikawa, 1987b). In the *Psila* larva, the dome of the ampullaceous sensillum of group B is perforated by pores above branched dendrites (Behan, unpublished results), which is consistent with an olfactory function. The recording electrode just penetrated the cuticle of this ampullaceous sensillum (3 μm diameter) so the records obtained may be viewed as single unit responses, which we measured as DC changes; the primary electrical event in the response of a chemosensory neuron is the DC generator or receptor potential. The reference electrode was inserted into the hemolymph adjacent to the cephalic lobes.

As electrophysiological responses diminished throughout an experiment, the response amplitudes to the test compounds were expressed as a percentage of the responses to a standard: 0.1 μg limonene for carrot root fractions and 0.5 μg *trans*-2-hexen-1-ol for the single compounds.

Consistent physical conditions are necessary in order to obtain reproducible electroantennograms (Adler, 1971). Thus, the cartridge was always positioned at the same distance (2 cm) from the preparation, the size of the filter paper within the cartridge was always identical, all cartridges had identical dimensions, and the flow rate of air was always the same (1 liter/min).

RESULTS

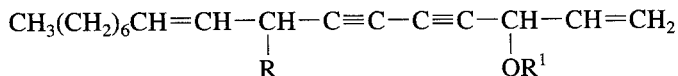
Extraction and Behavioral Assays of Root Volatiles. In each of four comparisons of total volatiles released into the headspace, resistant Vertou yielded less than susceptible Long Chantenay (Table 1). Tenax was threefold more

efficient than Porapak as adsorbent, and washes with diethyl ether yielded 8.8-fold more volatiles than subsequent washes with methanol, so the ether wash of the Tenax extraction is the most appropriate one for comparison. In this, total volatiles released by Vertou were less than one third the quantity released by Long Chantenay (Table 1).

Behavioral assays were made with a range of extracts including steam distillates, hexane-water, and chloroform-water extracts derived from Clause's Original Sytan (resistant), Long Chantenay (susceptible), and Danvers Half Long 126 (intermediate in resistance) (0.05–0.1 mg/disk). The aqueous layers from these extracts of the cultivars were all inactive. Only the steam distillate of Sytan and Danvers (5 μ l/disk), the hexane extract of Long Chantenay (0.1 mg/disk), and the chloroform extract of Sytan (0.05 mg/disk) were significantly preferred to their corresponding controls. Batches of Vertou and Long Chantenay extracted and fractionated in methanol (Figure 1) elicited too few significant responses to merit further detailed investigation.

In contrast, ether extracts were quite active, especially at 1 mg/disk. Although the essential oil extract of Vertou was inactive, that from Long Chantenay elicited a highly significant positive response (details of subfractions, yields, and responses in Figure 2). Neither the alcohol nor more polar fractions, collectively representing 26% and 60% of Vertou and Long Chantenay extracts, respectively, elicited any significant responses. In contrast, the fractions rich in hydrocarbon and carbonyl compounds elicited significantly positive responses (Figure 2).

As α -ionone, β -ionone, and bornyl acetate are attractants (Ryan and Guerin, 1982) and contain a carbonyl group, we focused on the carbonyl-rich fractions that were further fractionated by repeated silica gel chromatography monitored by thin-layer and gas-liquid chromatography (Figure 4). Elution by hexane-diethyl ether 19:1 followed by 4:1 afforded falcarinol (carotatoxin); further elution with the same solvents in the ratios 2:1 and 1:1 afforded falcarindiol monoacetate and falcarindiol (Figure 4). These compounds are represented by



where for falcarinol (carotatoxin) $\text{R} = \text{R}^1 = \text{H}$; for falcarindiol monoacetate $\text{R} = \text{OH}$, $\text{R}^1 = \text{Ac}$; and for falcarindiol $\text{R} = \text{OH}$, $\text{R}^1 = \text{H}$ (Figure 4). Of the three compounds, falcarindiol monoacetate is newly recorded from carrot. For completeness, the foliage of the two cultivars was subjected to an identical extraction, which also afforded falcarindiol monoacetate and falcarindiol but not falcarinol (Figure 4). Quantitatively, these three compounds collectively

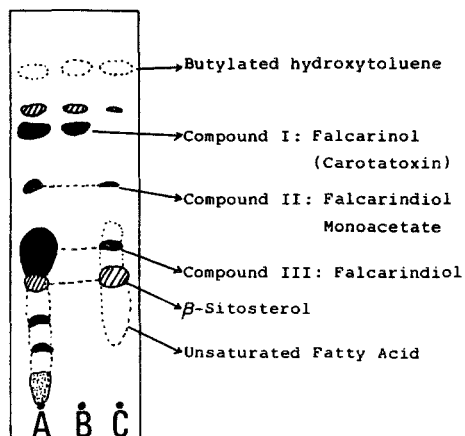


FIG. 4. Preparative thick-layer chromatography, developed in hexane-ethylacetate (1:1), of extracts of Long Chantenay roots with foliage included for completeness: (A) carrot root, ether extract; (B) carrot root, carbonyl fraction; (C) carrot foliage, ether extract. Compound I, a pale yellow oil, IR and [^1H]NMR values identical with those for falcarinol, carotatoxin (Crosby and Aharonson, 1967). Compound II, A colorless oil, falcarindiol monoacetate. [^1H]NMR CDCl_3 δ : 0.882 (3, t, $J = 6.7$, CH_3), 1.254 [2, s, $(\text{CH}_2)_6$],

H
|

2.105 (3, s, $-\text{OAC}$), 1.604 [2, 3 $-\text{C}_2$], 5.19–5.93 (5, m, $-\text{CH}=\text{CH}$, $-\text{CH}=\text{CH}_2$). Compound III, a pale yellow oil, falcarindiol, [^1H]NMR CDCl_3 δ : 0.9 (t, $J = 6.0$, CH_3), 1.30 [br, $(\text{CH}_2)_3$], 2.15 (m, OH), 5.0–6.2 (M) (olefinic protons).

represented 8.0% and 5.4% of the Long Chantenay and Vertou ether extracts, respectively, comprising 1.5 and 1.4% falcarinol, 5.9% and 3.6% falcarindiol, and 0.7 and 0.4% falcarindiol monoacetate, respectively. A check on the reliability of these values is provided by the measurement of the concentration of butylated hydroxytoluene, a commercial additive to stabilize ether, as 2.6% in the extract of both cultivars (mean of two replicates differing by less than 5%).

In behavioral assays, responses to falcarinol were significantly positive at 0.05 mg/disk and very highly significant at 0.10 mg/disk (Table 2). The positive response to β -sitosterol glucoside was significant at 0.01 mg/disk: scopletin and β -sitosterol were inactive. (\pm)-2-Methoxy-3-*sec*-butylpyrazine elicited highly significant positive responses at concentrations ranging from 0.05 to 10 mg/disk. As a check on the Petri dish method, bornyl acetate, established as an attractant following detailed behavioral observations including larvae-track records, was also assayed. It elicited very highly significant positive responses at 0.10 and 10.00 mg/disk (Table 2), thus confirming the utility of this method.

TABLE 2. RESPONSES OF CARROT FLY LARVAE TO INDIVIDUAL COMPOUNDS OCCURRING IN CARROT ROOT USING PETRI DISH METHOD

Compound	Conc. (mg/disk)	Larvae		R^a	t value	Significance
		Test	Control			
Falcarinol	0.05	44	24	29	2.43	$P < 0.05$
	0.10	63	24	45	4.18	$P < 0.001$
(\pm)-2-methoxy-3-sec-butylpyrazine	0.005	29	22	14	0.98	NS
	0.01	20	17	8	0.49	NS
	0.05	59	20	49	4.39	$P < 0.001$
	0.10	56	15	58	4.87	$P < 0.001$
	10.00	29	8	57	3.45	$P < 0.01$
Coumarin/scopeletin ^b	0.05	35	24	19	1.43	NS
	0.10	37	38	-1	-0.12	NS
	10.00	12	12	0.0	0.00	NS
β -Sitosterol ^b	0.10	34	27	11	0.89	NS
β -Sitosterol ² glucoside ^b	0.005	38	23	25	1.58	NS
	0.01	49	27	29	2.52	$P < 0.05$
	0.10	38	37	1	0.12	NS
Bornyl acetate	0.10	67	14	65	5.89	$P < 0.001$
	10.00	38	8	65	4.46	$P < 0.001$

^a R (%) = $(T - C/T + C) \times 100$ and rounded to nearest integer (details in text); negative values indicate negative responses.

^bDerived as in Figure 4.

Electrophysiological Assays. The ether-extracted essential oil of chopped Long Chantenay root was a potent stimulus, eliciting a peak response equivalent in amplitude to 250% that of the standard (Figure 5); the corresponding oil from Vertou was not available for this assay. The carbonyl fractions from Vertou and Long Chantenay were also active electrophysiologically but never exceeded 150% of standard response and so were less potent than the essential oil of Long Chantenay. The carbonyl fraction of Vertou was consistently less potent than that from Long Chantenay, and the lowest source concentration evincing clear separation in their effect was 0.01 μ g (Figure 5). Here, the response to Vertou was 45% weaker than standard, whereas that to Long Chantenay was 17% stronger than standard, or the carbonyl fraction of Vertou was about one half as potent a stimulus as that from Long Chantenay (Figure 5). The major substituents of the carbonyl-rich fraction, falcarinol and falcarindiol, were potent stimuli, eliciting at 10 ng a maximum response more than fivefold more potent than that of the standard; falcarindiol monoacetate was about 3.5-fold more potent (Figure 6).

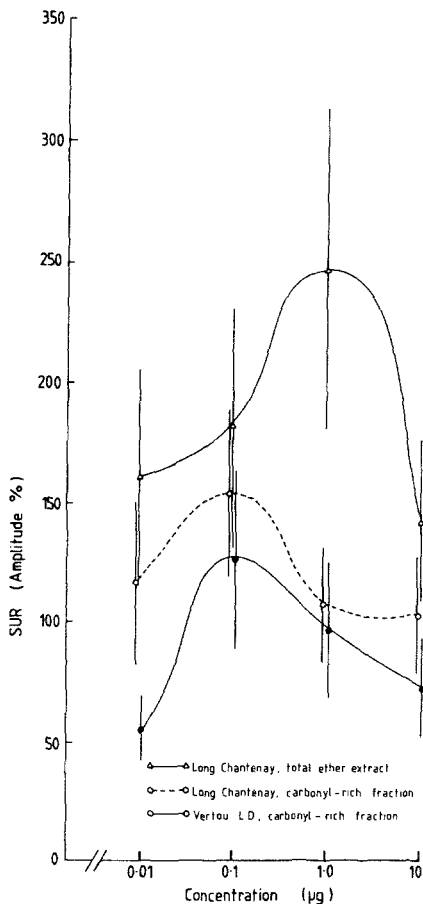


FIG. 5. Single unit responses (DC), from the group B ampullaceous sensillum of the *Psila* third-instar larva, to various concentrations of root extracts from Vertou and Long Chantenay. Responses are expressed as a percentage of the response to a standard, 0.1 μg limonene ($N = 5$; vertical bars here and in Figure 6 represent standard deviations).

DISCUSSION

The headspace comparison principally demonstrates a substantially decreased volatile release from pureed roots of resistant Vertou as compared with susceptible Long Chantenay. This is consistent with the earlier demonstration of decreased volatile release by the roots of resistant *Regulus Imperial*

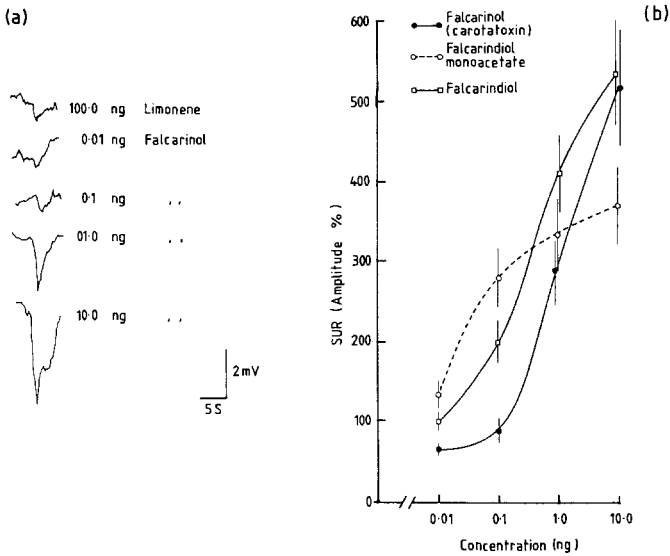


FIG. 6. (a) Unretouched traces of single-unit responses (DC) to various source concentrations of falcarinol (carotatoxin) with limonene included as standard. (b) Single unit responses (DC) from the group B ampullaceous sensillum of the *Psila* third-instar larva to various concentrations of the three constituents of the carotatoxin complex. Responses are expressed as a percentage of the response to a standard, $0.5 \mu\text{g}$ *trans*-2-hexen-1-ol ($N = 5$).

as compared with susceptible Chantenay-Red Cored-Elite (Guerin and Ryan, 1984). Accordingly, such decreased release by carrot root may be a significant feature of *Daucus* resistance to *Psila*. The adsorbent superiority of Tenax over Porapak also seems noteworthy.

Ether extracts of chopped root were the most potent, and the principal component of their strongly preferred carbonyl-rich fraction was the carotatoxin complex comprising falcarinol, falcarindiol, and falcarindiol monoacetate. This seems to be the first report from carrot of the monoacetate, which we also identified in the extracted foliage of each cultivar; an earlier investigation of foliage did not detect this compound (Städler and Buser, 1984).

In quantitative terms, the larger yield of ether-extracted oil from Long Chantenay compounded by the larger percentage concentration represented by the toxin complex in Long Chantenay produces an increased amount of 13.07 ppm, or $1046 \mu\text{g}$ toxin complex per typical root of 80 g. In regard to individual constituents of the complex, the variation in the percentage concentration represented by falcarinol would seem to be close to error limits. However, the

additional yield of Long Chantenay oil would indicate additional amounts of 70 μg falcarinol per Long Chantenay root; corresponding increased amounts for falcarindiol and falcarindiol monoacetate are 862 μg and 110 μg , respectively.

The extra 70 μg falcarinol per Long Chantenay root exceeds the minimum source concentration of 50 μg falcarinol that elicited a significantly positive behavioral response from the larva. Increasing the source from 50 to 100 μg strengthened this response, so the 70 μg excess falcarinol seems behaviorally relevant. Furthermore, as the smallest of these additional amounts is 70,000 times larger than the source amount eliciting maximal electrophysiological response from the larva, the extra amount of toxin complex also seems relevant at the neurophysiological level. Given that the five individual compounds previously identified as larval attractants collectively constitute not more than 3 μg /root (Ryan and Guerin, 1982), they now seem less significant than the toxin complex as larval attractants and as resistance factors.

In a comparison of six carrot leaf substituents, falcarindiol was the most potent stimulus for oviposition by *Psila* and the only constituent active at concentrations equal to or less than those in the leaf surface (Städler and Buser, 1984). The estimated threshold concentration for egg laying was 100 ng falcarindiol and each 6 g of leaf contained about 3200 ng of this compound (Städler and Buser, 1984). We estimate that 6 g of the average root of Vertou and of Long Chantenay would contain 69 μg and 134 μg falcarindiol. Accordingly, the root moiety of this compound could surpass that in the leaf as an egg-laying stimulus. Furthermore, the additional amount in a Long Chantenay root (65 μg /6 g or 862 μg /root) seems more than sufficient to affect the differential egg laying we observed as influenced by root (Maki and Ryan, 1989). So there can be little doubt that falcarindiol is a crucial compound in the relationship between *Psila* and its host by serving as: (1) the principal reported stimulus for oviposition, (2) a potent neurophysiological stimulus for the larva, and (3) a contributing factor, through decreased concentrations, to the superior root resistance of Vertou.

When injected into mice, falcarinol (carotatoxin) evokes neurotoxic symptoms with an LD value of ca. 100 mg/kg body weight and is also toxic to the crustacean, *Daphnia magna* (Straus) (Crosby and Aharonson, 1967), effects that have been assigned to falcarindiol by Städler and Buser (1984) [antifungal properties have been demonstrated for falcarindiol (Kemp, 1978)]. Falcarinol was first given the trivial name "carotatoxin" by Crosby and Aharonson (1967), and subsequently the compound was confirmed as falcarinol (Bentley and Thaller, 1967), the polyacetylenic alcohol first isolated from *Falcaria vulgaris* Bernh. (Bohlman et al., 1966). Thus, a neurotoxin affecting vertebrates and invertebrates, and presumably capable of inhibiting feeding by them, would seem to have become a host-location cue for the *Psila* larva, which is consistent with the coevolutionary concept of plant-insect relationships. It is appropriate

to contrast this evolutionary response to the toxins with the failure of *Psila* to similarly evolve to another toxic ingredient of carrot root, *trans*-2-nonenal, which repels, paralyzes, and kills the larva (Guerin and Ryan, 1980). As *trans*-2-nonenal comprises only 40 μg /average carrot root, in contrast to 2394 μg represented by the toxin complex, the larger concentration of toxin might more readily elicit adaptation.

It has been reported that carrot cultivars more susceptible to root fly damage contain larger concentrations of chlorogenic acid, the principal phenolic constituent of carrot root peel (Cole, 1985). However, the fact that carrots overwintering in soil and subject to damage by frost also exhibited elevated levels of chlorogenic acid suggested that any damage to the root elicits this response (Cole, 1985). Subsequent observations did not experimentally separate "cause" and "effect" in this relationship (Cole et al., 1987).

The present data indicate that decreased levels of the carotatoxin complex confer resistance on carrot roots by making them less attractive to larvae. Although there are other examples of plant toxins employed as host-location cues of insect parasites, this seems a relatively rare example of decreased concentrations of a neurotoxin eliciting nonpreference resistance.

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RUFFED GROUSE FEEDING BEHAVIOR AND ITS RELATIONSHIP TO SECONDARY METABOLITES OF QUAKING ASPEN FLOWER BUDS¹

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Abstract—Quaking aspen (*Populus tremuloides* Michx.) staminate flower buds and the extended catkins are primary food resources for ruffed grouse (*Bonasa umbellus*). Winter feeding observations indicate that ruffed grouse select specific trees or clones of quaking aspen to feed in. Flower buds and catkins of quaking aspen were analyzed for secondary compounds (tannins, alkaloids, and phenolics) that might cause ruffed grouse to avoid trees with high levels of these compounds. Coniferyl benzoate, a compound that has not been previously found in quaking aspen, exists in significantly higher concentrations in buds from trees with no feeding history as compared to ruffed grouse feeding trees. Aspen catkins were also significantly lower in coniferyl benzoate than buds from the same tree. Ruffed grouse feeding preference was not related to the tannin or total phenolic levels found in buds or catkins. Buds from feeding trees had higher protein levels than trees with no feeding history; however, catkins did not differ from buds in protein concentration. The high use of extended catkins in the spring by ruffed grouse is probably due to a lower percentage of bud scale material in the catkin as opposed to the dormant bud. Bud scales contain almost all of the nontannin phenolics in catkins and dormant buds. A feeding strategy where bud scales are avoided may exist for other bird species that feed on quaking aspen. Dormant flower buds are significantly lower in protein-precipitable tannins than catkins and differ in secondary metabolite composition from other aspen foliage.

Key Words—Coniferyl benzoate, *Populus tremuloides*, *Bonasa umbellus*, Fringillidae, feeding behavior, secondary metabolites, tannins, phenols.

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INTRODUCTION

The importance of quaking aspen (*Populus tremuloides* Michx.) in the diet of ruffed grouse (*Bonasa umbellus*) has been recognized by numerous investigators (Svoboda and Gullion, 1972). Ruffed grouse rely on quaking aspen as a primary food source throughout the year; however, their dependence on this species is most evident during the winter and early spring when their alternative food sources are limited (Gullion, 1966; Vanderschaegen, 1970; Svoboda and Gullion, 1972; Doerr et al., 1974; Huempfer, 1981). The item that ruffed grouse consume most frequently during the winter is the staminate flower bud of quaking aspen (Vanderschaegen, 1970; Svoboda and Gullion, 1972; Doerr et al., 1974). Use of the staminate flowers continues in the spring, when, for a limited time, ruffed grouse exist on almost an exclusive diet of the extended catkins (Gullion, 1964; Vanderschaegen, 1970; Stoll et al., 1980).

Although quaking aspen is one of the primary food resources for ruffed grouse, feeding observations indicate that ruffed grouse normally feed only in certain trees or clones of quaking aspen and that preference for certain trees may change over time (Gullion, 1966; Huff, 1970; Schemnitz, 1970; Huempfer, 1981). Characteristically, ruffed grouse feed in older trees (30–50 years old) that are physiologically stressed (Huff, 1970; Svoboda and Gullion, 1972; Doerr et al., 1974). The preferential use of certain clones and trees can be explained partially by ruffed grouse selecting trees that have buds with higher nutrient levels; however, the periodic lack of use of quaking aspen buds by ruffed grouse cannot be readily explained by fluctuating nutrient levels (Huff, 1970; Doerr et al., 1974).

Long-term studies of ruffed grouse populations indicate that their populations cycle every 8–10 years, especially in the northern latitudes of their range (Keith, 1963; Gullion, 1970, 1984). Researchers in Minnesota and Alberta have noted that sharp declines in ruffed grouse populations and their periodic low population levels have coincided with the lack of use of aspen buds during the winter (Gullion, 1970, 1984; Doerr et al., 1974; Huempfer, 1981; D. Rusch, University of Wisconsin, unpublished data). Servello and Kirkpatrick (1987), sampling grouse crop contents during periods of depressed populations, found few aspen buds among birds coming from regions (e.g., Maine, Wisconsin, New York) where there has been heavy use of these buds at other times (Brown, 1946; Bump et al., 1947; Stollberg and Hine, 1952). This decrease in the use of quaking aspen can occasionally be attributed to a failure in the aspen bud crop; however, during many of these years the aspen bud crop has been ample (Doerr et al., 1974; Gullion, 1977, 1984, and unpublished data).

One possible explanation for the differential use of aspen buds during the ruffed grouse population cycle is that the overall nutritive value of the buds changes (Lauckhart, 1957). Studies on how the standard nutrients in quaking

aspen vary with cyclic animal populations indicate that nutrient levels do not track these cyclic populations (Pease et al., 1979; Batzli, 1983). However, the overall nutritional value of food is only partially determined by standard nutrients. Secondary compounds also play an important role in determining the value of a plant as a food source (Bryant and Kuropat, 1980; Bryant, 1981; Rhoades, 1983; Reichardt et al., 1984). Gullion (1977) attributed the lack of use of quaking aspen buds by ruffed grouse, during several consecutive years of abundant bud crops, to some type of "plant defensive mechanism." Additionally, he noted that the degree of aspen bud use by ruffed grouse was later reflected in their population levels. This periodic decrease in use of flower buds contrasts to the consistently high use of the catkins from these buds in the spring (Gullion, 1984). Previous investigations do not clearly indicate whether nutrient levels change significantly as aspen buds mature into catkins and how these changes are related to ruffed grouse selection for this item.

The objectives of this study were twofold. The first objective was to obtain preliminary information on the secondary chemistry of quaking aspen staminate flower buds and catkins, with the intent of identifying the compounds that would most likely act as primary feeding deterrents towards ruffed grouse. The second objective was to test the hypothesis that ruffed grouse feeding preference for quaking aspen catkins and certain flower buds is related to differential levels of specific secondary compounds.

METHODS AND MATERIALS

Sample Collection and Storage

Ruffed grouse feeding behavior was observed three times a week, from December to April (1985 and 1986), at the Mille Lacs Wildlife Management Area, in east-central Minnesota and at the Cloquet Forestry Center located near Duluth, Minnesota. Feeding observation methods are detailed in Svoboda and Gullion (1972). Quaking aspen that ruffed grouse were observed feeding in were designated as "preferred" trees and marked.

Quaking aspen buds were sampled from mid-January through the first week in March (1985 and 1986) at the Mille Lacs and Cloquet study sites. Samples were collected during the period when ruffed grouse normally make the heaviest use of quaking aspen buds and when physiological changes in these buds are relatively minor (Huff, 1970; Huempfer, 1981). When a preferred tree was sampled, an additional quaking aspen with no feeding history (nonpreferred tree) was sampled and treated as a paired tree. Trees were considered paired due to similar site characteristics and time of sampling. In order to minimize chemical variation between paired trees, nonpreferred trees were chosen from the same age class as preferred trees, and only the upper crowns of each tree

were sampled. To assure clonal separation of paired trees, nonpreferred trees were chosen at least 200 ft from preferred trees, and distinguishing characteristics of the preferred tree's clone were noted (Blake, 1963; Barnes, 1966). A total of 11 pairs of preferred and nonpreferred trees were sampled during the two field seasons.

After the branches were brought to the ground by shooting them with a small bore rifle, staminate flower buds were immediately picked, put into air tight plastic bags or Nalgene (Nalge 2007) bottles and stored at approximately -50°C on Dry Ice (Bielecki, 1964; Robinson, 1980; Bryant et al., 1983). Enzymes such as polyphenol oxidase can readily change phenolic compounds into quinones and other oxidation products (Takai and Hubbes, 1973; William Gordon, Howard University, personal communication; Mattes et al., 1987). Therefore, samples were kept on Dry Ice until they were lyophilized, after which they were ground with a mortar and pestle under liquid nitrogen and stored at -18°C (Swain, 1979; Martin and Martin, 1982).

Catkin sampling procedures were essentially the same as for dormant winter buds. Bud scales normally adhere to the base of the catkin after the catkins are picked from the branches. These scales were included in the catkin samples, since bud scales were seen in droppings from ruffed grouse that had been feeding on catkins. A total of 18 nonpreferred trees were sampled for their dormant buds and again in mid-April when their catkins had extended. Since ruffed grouse consistently utilize catkins, whether or not they have made use of the dormant winter buds, catkin samples were considered preferred food items. Therefore, bud and catkin samples from individual nonpreferred trees were treated as paired nonpreferred and preferred samples respectively.

Chemical Analyses

Tannins. Aspen bud and catkin samples were analyzed for both protein-precipitable tannins and condensed tannins. Protein-precipitable tannins were measured using the radial diffusion assay (Hagerman, 1987). Samples (100 mg) were extracted in acetone and water (70:30) (5.00 ml) for three days. A calibration curve was constructed using purified tannic acid (courtesy of A.E. Hagerman) and used to calculate the tannic acid equivalents in the extracts.

Condensed tannins were measured using a modified form of the proanthocyanidin assay of Watterson and Butler (1983). Samples (200 mg) were extracted in 2.00 ml of acidic methanol (1% HCl v/v) for 1 hr. The extract (0.20 ml) was dispensed into a test tube, and 2.00 ml of acidic butanol (30% HCl v/v) was added. This mixture was heated in a boiling water bath for 10 min, after which its absorbance was measured at 550 nm on a Beckman DB GT spectrophotometer (A.E. Hagerman, personal communication).

Alkaloids. Aspen bud samples were extracted following the methods outlined in Lindroth et al. (1986). Alkaloid content was analyzed by thin-layer chromatography (TLC) using silica gel plates (Kodak 13179) that were developed in toluene-chloroform-acetone (90:30:10) and visualized with Dragendorff Munier-Macheboeuf reagent or iodine-iodide reagent (Santavy, 1969; Touchstone and Dobbins, 1978). Isoniazid (Aldrich Chem. Co.), a pyridine alkaloid found in quaking aspen leaves, was used as a standard and cochromatographed with the bud extracts (Sunarjo, 1985). Visualization reactions were carried out both on TLC plates and standard filter paper due to an inverse reaction (light spot instead of dark) of the visualization reagents with isoniazid on the silica gel plates.

Phenols. Specific phenolic compounds and total phenolics were both investigated. Total phenolics were estimated using the Prussian blue assay (Price and Butler, 1977). Samples (0.20 g) were extracted in 2.0 ml of acidic methanol (1% HCl v/v) for 1 hr. Ferric ammonium sulfate was substituted for ferric chloride in the reaction, and potassium ferricyanide was added 20 min after the ferric ammonium sulfate addition (A.E. Hagerman, personal communication). Absorbance was measured at 720 nm on a Beckman DB GT spectrophotometer. A calibration curve was constructed using commercial gallic acid and was used to calculate gallic acid equivalents in the samples.

Bud samples that were qualitatively analyzed by TLC were extracted overnight (500 mg dry wt) in 3 ml of reagent grade methanol. The phenolic compounds that were analyzed by high-performance liquid chromatography (HPLC) were relatively nonpolar; therefore, ethyl ether was used as the extraction solvent. Bud and catkin samples (100 mg dry wt) were extracted for 24 hr in 5.0 ml of anhydrous ethyl ether, after which the eluent was drawn out of the plant material with a Pasteur pipet, and concentrated in vacuo to 1 ml. The extract was subsequently stored at -18°C until analyzed.

Initial qualitative and semiquantitative comparisons between preferred and nonpreferred samples were done using TLC. Silica gel (Kodak) plates were developed in either toluene-chloroform-acetone (40:25:35), toluene-acetone-formic acid (46:46:8), or chloroform-methanol-water (80:19:1), depending on the polarity of the compounds of interest (Egger, 1969; Lea, 1978). Aspen buds were tested for simple phenolics and phenolic glycosides including: salicortin, salicin, tremulacin, tremuloiden, *p*-coumaric acid, catechol, and benzoic acid. Compounds from aspen bud extracts that had similar R_f values as commercial standards were scraped from the TLC plate and rechromatographed with commercial standards on Merck reversed-phase, high-performance thin-layer plates (HPTLC) (Fried and Sherma, 1982). Plates were developed in aqueous 0.5 M sodium chloride-methanol (50:50) (Fried and Sherma, 1982).

Quantitative analyses were performed using HPLC. Pinosylvin dimethyl

ether (PDE) was used as the internal standard. Aspen bud and catkin extracts were diluted with 4.00 ml of methanol containing 150 μg of PDE. Samples were injected (20 μl) on an analytical column (25 cm \times 4 mm) packed with 10 μm Waters gamma bondapak C_{18} reverse-phase sorbent. The gradient program was: T1 = 0 min, 42% A, ramp time 1 min; T2 = 25 min, 60% A, ramp time 1 min; T3 = 40 min, 42% A, flow rate 1 ml/min; where solution A was acetonitrile and solution B 2% tetrahydrofuran in water. A Schoeffel SF770 and GM770 variable wavelength detector, set at 295 nm was used for detection of sample components. Peaks were quantified using peak height measurements that were standardized to peak areas of the internal standard and the compounds of interest. Reproducibility was within 1%.

Isolation of the suspected feeding deterrent was accomplished by extracting aspen bud samples (1 g) with reagent grade methanol (5 ml) for 24 hr. Methanol appeared to be equal to ether in its extraction efficiency, and its polarity made it superior to ether for large sample injections on the HPLC. Glass containers were used throughout the isolation procedure to prevent phthalate contamination. The suspected feeding deterrent was separated from the crude bud extract by HPLC. HPLC conditions were the same as previously mentioned with the exception that 50–100 μl of extract was manually injected, and detection was made with a diode array 1040M Hewlett Packard detector in conjunction with a HP9133 Analytical Chemstation. After a sufficient amount of sample was collected in a darkened test tube, the acetonitrile in the eluent was evaporated by vortexing the sample under a stream of nitrogen. The remaining sample was then extracted with HPLC-grade ethyl ether, dried (MgSO_4), and concentrated under a stream of nitrogen.

Mass spectra of the isolated compound were run on a Kratos MS25 with a Data General S/120 data system using direct probe insertion, at 70 eV. Samples for [^1H]NMR analyses were prepared by evaporating the ether from the isolation sample under a stream of nitrogen and redissolving the sample in D -chloroform. [^1H]NMR spectra were run on a Nicolet NT 300 MHz, wide-bore, Fourier transformer NMR. Nuclear Overhauser enhancer (NOE) and selective decoupling experiments aided in structure determination.

Nutrient Analyses. Methods for analyzing nutrients were chosen to duplicate those of Huff (1970), so that direct comparisons could be made between his study and ours. Buds and catkins were analyzed for Kjeldahl nitrogen content using the methods outlined in Bradstreet (1965) by the University of Minnesota's Department of Soil Science, Research Analytical Laboratory. Protein levels were calculated by multiplying the Kjeldahl nitrogen content by 6.25 (Huff, 1970). Crude fat, or ether-extractable material, was determined according to method 20.034 in the Association of Official Agricultural Chemists (1965).

RESULTS

The tannin and total phenolic content of the paired preferred and nonpreferred bud samples appear to have been affected by storage conditions (samples may have thawed). Relative comparisons between paired preferred and nonpreferred bud samples should be valid since storage conditions were identical for both preferred and nonpreferred buds, and any degradation that occurred should have occurred equally in both types of samples. However, true tannin and total phenolic levels for these samples may be underestimated. Paired catkin and bud samples were all collected the following year and do not appear to have suffered any sample degradation. The 1986 samples were compared to bud samples that were analyzed immediately after collection and were found to have condensed tannin and total phenolic values equivalent to samples that were analyzed immediately.

Tannins. Quaking aspen buds had significantly lower levels of protein-precipitable tannins than catkins from the same trees (Tables 1 and 2). Comparisons could not be made between paired preferred and nonpreferred dormant buds, since the majority of these samples were below the detection threshold.

TABLE 1. SUMMARY OF QUANTITATIVE TESTS COMPARING SECONDARY CHEMISTRY AND NUTRIENTS OF PREFERRED (P) AND NONPREFERRED (NP) QUAKING ASPEN BUDS AND CATKINS

Parameter measured	Sample comparison	Statistical tests	Sample size ^a	P value
Protein-Precipitable tannins	NP buds vs. catkins ^b	Paired <i>t</i> test	14	0.001
Condensed tannins	NP buds vs P buds	Paired <i>t</i> test	9	0.345
	NP buds vs. catkins	Paired <i>t</i> test	14	0.740
Total phenolics	NP buds vs P buds	Paired <i>t</i> test	9	0.184
	NP buds vs. catkins	Paired <i>t</i> test	14	0.224
Coniferyl benzoate	NP buds ^b vs. P buds	Paired <i>t</i> test	11	0.008
	NP buds ^b vs. catkins	Paired <i>t</i> test	18	0.006
Protein	NP buds vs. P buds ^b	Paired <i>t</i> test	9	0.062
	NP buds vs. catkins	Paired <i>t</i> test	10	0.647
Crude fat	NP buds vs. P buds	Welch <i>t</i> test ^c	10, 5	0.914
	NP buds ^b vs. catkins	Paired <i>t</i> test	10	0.042

^aSample size for paired *t* test indicates number of paired samples.

^bIndicates sample having the highest concentration for tests where the significance level was less than or equal to 0.1.

^cOtt (1984).

TABLE 2. MEAN TANNIN AND TOTAL PHENOLIC LEVELS IN PREFERRED (P) AND NONPREFERRED (NP) QUAKING ASPEN FLOWER BUDS AND CATKINS

Sample type	Total phenolics ^a (% dry wt)	Protein-precipitable tannins ^b (% dry wt)	Condensed tannins (A550/g)
P buds ^c	1.44 ± 0.22	ND ^d	3.10 ± 0.80
NP buds ^c	1.72 ± 0.18	ND	3.68 ± 0.64
Catkins	1.95 ± 0.15	2.68 ± 0.29	7.77 ± 0.91
NP buds ^e	2.30 ± 0.16	1.43 ± 0.088	7.39 ± 0.59

^aReported in equivalent units of gallic acid.

^bReported in equivalent units of tannic acid.

^cPhenolic and tannin levels are lower due to storage conditions.

^dNot determined due to low concentrations.

^ePaired with catkin samples.

Condensed tannin levels were not significantly different between paired preferred and nonpreferred buds, nor was there a significant difference between buds and catkins from the same tree (Table 1). The mean condensed tannin levels for buds and catkins were relatively low as compared to trees more noted for their "tannin defense" (Table 2). Using white oak foliage (*Quercus alba* L.) for a relative comparison, quaking aspen buds and catkins had approximately 13% of the condensed tannins found in *Q. alba* foliage (Martin and Martin, 1982).

Alkaloids. TLC analyses indicated that quaking aspen buds did not contain any detectable alkaloids. Fairly minute quantities of alkaloids should have been detected by TLC. Samples were concentrated to the point that if isoniazid was present at the same concentration as found in aspen leaves (0.036% fresh weight), each chromatographic spot would have contained approximately 2.16 µg of isoniazid, which is far above the detection limit for most alkaloids (Santavy, 1969; Lindroth et al., 1986).

Phenolic Compounds. The functional group assay for total phenolics did not indicate that aspen buds differ significantly from catkins in total phenolics (Table 1). Likewise, no significant difference was found between preferred and nonpreferred buds in total phenolics (Tables 1 and 2).

Thin-layer chromatographic analyses indicated that quaking aspen buds contained salicin (R_f 0.17) and salicortin (R_f 0.17–0.18) (chloroform–methanol–water 80:19:1) in relatively low concentrations and did not contain the phenolic glycosides tremuloiden and tremulacin, which have been reported in quaking aspen foliage (Pearl and Darling, 1971; Lindroth et al., 1987). Catechol (R_f 0.58) and *p*-coumaric acid (R_f 0.25) both appeared to be present when initially analyzed by TLC. However, only *p*-coumaric acid (R_f 0.49) was confirmed by

reversed phase HPTLC. It appeared that *p*-coumaric acid was present only in minute quantities after it was separated from its accompanying impurities by HPTLC. Benzoic acid could not be visualized by TLC; therefore, commercial benzoic acid and aspen bud extract were compared by HPLC. Benzoic acid eluted at 2.5 min, which matched the retention time of a compound in aspen buds. The UV spectra (analyzed by diode-array detector) of this compound was found to be identical to that of benzoic acid (Silverstein et al., 1974).

Preliminary TLC comparisons between paired preferred and nonpreferred bud and catkin samples indicated that there were three compounds that appeared to be present at higher levels in the nonpreferred samples. One of these compounds (R_f 0.52–0.54) (toluene–chloroform–acetone 40:25:35) appeared to be present at a higher concentration than any other secondary phenolic compound in quaking aspen buds and catkins. The other two compounds (R_f 0.34–0.38 and 0.74–0.78) were present in relatively small amounts. The high concentration of the compound at R_f 0.52–0.54 led to the assumption that this particular compound had the highest potential for exhibiting significant feeding deterrent properties.

Quantitative analyses using HPLC indicated that buds from nonpreferred and preferred trees had mean concentrations of the suspected feeding deterrent of 0.531 ± 0.086 (SE) and 0.251 ± 0.032 (SE) (% dry wt/PDE), respectively. Concentrations are expressed in relative amounts since a standard for the suspected feeding deterrent was not available. Buds from nonpreferred trees were significantly higher in concentrations of the suspected feeding deterrent than preferred trees (Table 1 and Figure 1). Likewise, analyses of paired bud and catkin samples indicated that buds had significantly higher amounts of the sus-

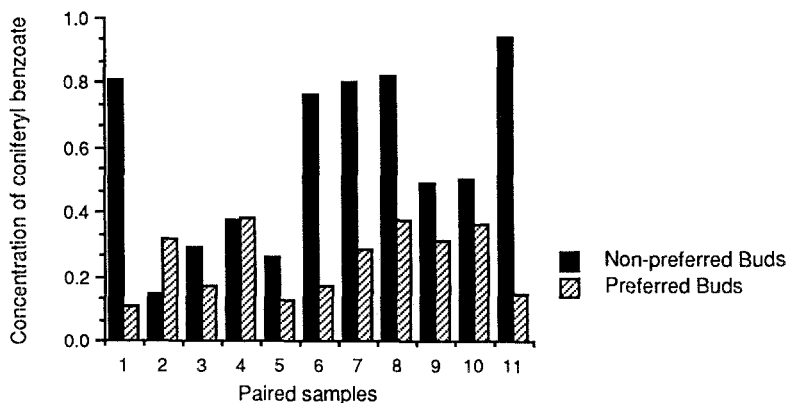
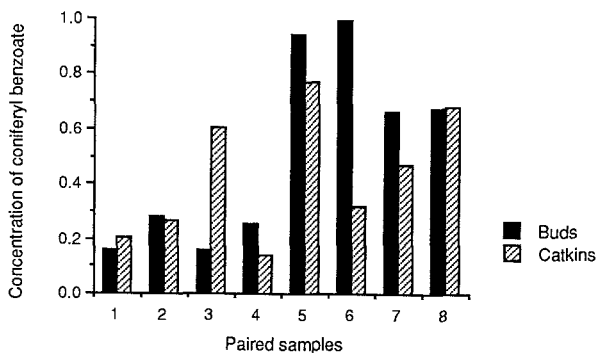


FIG. 1. Relative concentration (% dry wt/PDE) of conferyl benzoate for 1985 and 1986 preferred and nonpreferred staminate flower buds.

pected feeding deterrent than catkins (Table 1 and Figure 2). Mean concentrations of the suspected feeding deterrent in buds and catkins were 0.549 ± 0.59 (SE) and 0.355 ± 0.43 (SE) (% dry wt/PDE), respectively.

Structural analyses of the suspected feeding deterrent by $[^1\text{H}]\text{NMR}$ indicated that it is *trans*-coniferyl benzoate (Figure 3). Peak assignments were based on chemical shifts, double irradiation experiments, and comparison with spectra of authentic coniferyl benzoate (Kato, 1984). The location of the methyl was confirmed by a NOE experiment. Specifically, irradiation of the $-\text{OCH}_3$ at 3.91 ppm resulted in a large increase in the singlet located at 6.94 ppm but not

a.



b.

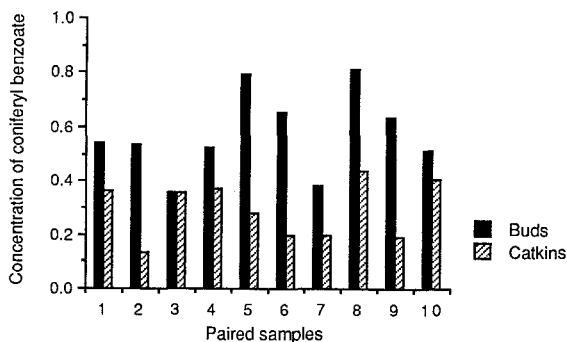


FIG. 2. Relative concentration (% dry wt/PDE) of coniferyl benzoate from 1986 buds and catkins collected from the (a) the Cloquet study site and (b) the Mille Lacs study site.

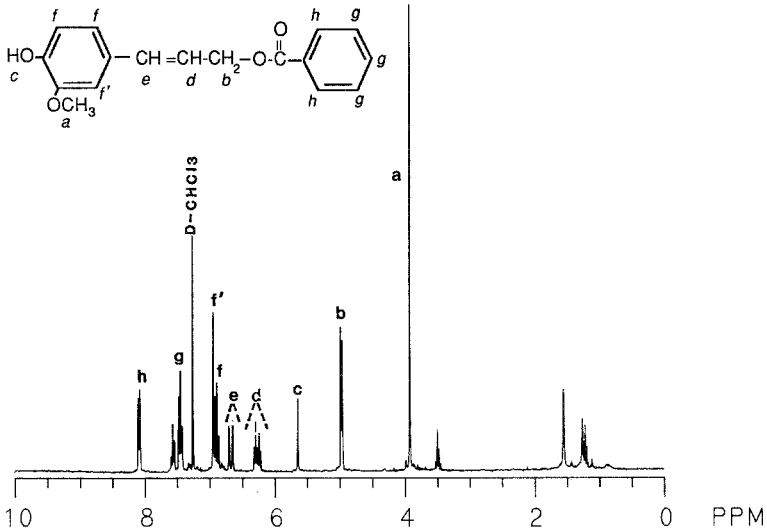


FIG. 3. $[^1\text{H}]$ NMR spectra of the suspected feeding deterrent (coniferyl benzoate).

in the apparent triplet located at 6.88 ppm, indicating that the methyl was nearest an unsplit aromatic hydrogen. The *trans* geometry was elucidated by coupling ($J = 15$ Hz) between the olefinic hydrogens. Finally, mass spectral analyses showed a molecular ion (284) in addition to fragment ions corresponding to benzoyl (105) and coniferyl (163) cations.

Nutrients. Protein levels were not significantly different between buds and catkins; however, preferred buds were found to have slightly higher levels of protein than nonpreferred buds (Tables 1 and 3).

TABLE 3. MEAN PROTEIN AND CRUDE FAT LEVELS IN PREFERRED (P) AND NONPREFERRED (NP) QUAKING ASPEN FLOWER BUDS AND CATKINS

Sample type	Protein (% dry wt)	Crude fat (% dry wt)
P buds	13.01 \pm 0.89	7.34 \pm 0.55
NP buds	10.66 \pm 0.63	
Catkins	9.88 \pm 0.65	5.21 \pm 0.53
NP buds ^a	9.49 \pm 0.48	7.46 \pm 0.83

^aPaired with catkin samples.

Crude fat levels were significantly lower in catkins than in nonpreferred buds, which is contrary to what would be expected for a preferred food item (Tables 1 and 3). Preferred and nonpreferred bud samples were not significantly different in their crude fat content (Tables 1 and 3).

The crude fat analysis (20.034 A.O.A.C.) gives only a rough estimation of the fat content of a sample, due to additional ether-soluble material being extracted along with the fat. This method is not well suited for aspen buds. The high drying temperatures and vacuum used in the procedure not only evaporate any residual water but also many of the volatile compounds. This results in wide variations in crude fat estimates, which are dependent on the exact drying time and conditions. Relative comparisons between samples are possible only if strict adherence to standardized drying conditions can be met. Comparing the crude fat analyses of aspen buds between different investigators, without knowledge of the specific drying conditions, would likely be meaningless.

DISCUSSION

Ruffed grouse feeding preference for both aspen buds and catkins was consistently related to the level of coniferyl benzoate found in these items. Although the overall mean concentration of coniferyl benzoate was lower in catkins as compared to buds, this pattern did not occur in every tree (Figure 2a). This apparent inconsistency may be due partly to differences in quaking aspen phenology at the time the catkins were collected. The ratio of bud scales to reproductive material in catkins is dependent upon the maturity of the catkin. A higher ratio of bud scales to reproductive material will effect the amount of coniferyl benzoate that is present in a gram of catkin material (see following paragraphs). Partially extended catkins coupled with within-tree variation of coniferyl benzoate levels may explain why some catkin samples had slightly higher levels of coniferyl benzoate than buds from the same tree. Even with these variations, catkins had significantly lower levels of coniferyl benzoate when compared with buds from the same tree. Similarly, a few nonpreferred trees had buds with lower levels of coniferyl benzoate than buds from preferred trees (Figure 1). All of these nonpreferred trees had relatively low levels of coniferyl benzoate, likely below the tolerance threshold of ruffed grouse for this compound, indicating that these trees were not selected as feeding trees for other reasons.

In order to assess why one food item is selected over another, the available nutrients in these items must also be taken into account. Ruffed grouse showed a preference for buds that were higher in protein in this study and in earlier investigations (Huff, 1970; Doerr et al., 1974). Although slightly higher protein levels may offer a partial explanation for the selection of aspen buds, protein

levels do not explain the general preference ruffed grouse have for aspen catkins. Aspen catkins do not differ significantly from buds in protein levels. This conclusion differs from those made by Huff (1970) on the nutritional differences between catkins and buds; however, Huff failed to test the significance of the differences he reported. He also did not compare buds and catkins from the same tree, which may have increased the amount of variance between samples.

One likely reason for the lower concentration of coniferyl benzoate in catkins is due to the relatively lower amount of bud scale material in the catkin as compared to the dormant bud. TLC analyses of bud scales and reproductive tissues of dormant buds and catkins indicate that virtually all of the nontannin phenolics are located in the bud scale material. The increased ratio of reproductive to bud scale material in the catkin would enable ruffed grouse to feed on a greater percentage of catkin material that is relatively free from coniferyl benzoate and other simple phenolic compounds. This would explain why ruffed grouse consistently feed on catkins even during years when they avoid the dormant buds.

The avoidance of flower bud scale material by an unknown species of bird has also been observed. While collecting bud samples, it was noted that branches from a particular tree had many staminate flower buds that had their centers removed (presumably consumed), yet the outer bud scales remained intact. A feeding strategy in which only the center portion of the bud was removed would allow a bird to avoid almost all of the simple phenolic compounds, particularly coniferyl benzoate, in the dormant winter bud. This method of feeding on quaking aspen buds cannot be attributed to ruffed grouse since ruffed grouse feed on quaking aspen buds by removing the entire bud. However, both the purple finch (*Carpodacus purpureus*) and evening grosbeak (*Coccothraustes vespertinus*), which feed on quaking aspen flower buds and are present in the study area during the winter, might exhibit this feeding behavior (Shaub, 1956; Bent, 1968; Gullion, 1985).

Huff (1970) compared levels of a number of other nutritional parameters between the buds of preferred and nonpreferred quaking aspen and concluded that buds from preferred trees had significantly lower levels of lignin and acid detergent fiber ($0.05 > P > 0.01$), along with higher levels of phosphorus, potassium, and moisture ($0.1 > P > 0.05$), than buds from randomly selected trees with no feeding history. Therefore, ruffed grouse feeding preference for buds from specific quaking aspen trees might be due to a combination of factors, including the levels of certain nutrients and allelochemicals. However, available data suggest that the level of coniferyl benzoate is one of the most significant differences between preferred and nonpreferred buds.

Servello and Kirkpatrick (1987) related the nutritional value of the natural foods of ruffed grouse, and their preference for these foods, to tannin and total phenolic levels in these items. Theoretically, total phenolics and tannins may

lower the nutritional value of foods; however, our study indicates that tannin content and total phenolics may not be reliable indicators of ruffed grouse feeding preference. Total phenolic concentration was not significantly related to ruffed grouse selection of aspen buds or to their general preference for aspen catkins. Total phenolic measurements were also not correlated with levels of coniferyl benzoate, even though coniferyl benzoate appears to be the primary simple phenol in aspen buds. Likewise, tannin levels (measured as protein-precipitable and condensed tannins) were not related to ruffed grouse feeding preference. Protein-precipitable tannins were significantly higher in aspen catkins, a preferred food item, as compared to nonpreferred buds. The level of tannins found in aspen catkins and buds (expressed in equivalent amounts of tannic acid) should have been sufficient to have a physiological effect on ruffed grouse (Vohra et al., 1966; Rostagno et al., 1973).

Several explanations can be postulated for this lack of correlation between tannin levels and feeding preference. Many herbivores are thought to have evolved adaptations to handle secondary compounds common in their diets (Rhoades, 1985; Robbins et al., 1987). The diet of ruffed grouse likely contains a number of items with tannin levels similar to the levels found in aspen catkins (Servello and Kirkpatrick, 1987). Ruffed grouse may have physiologically adapted to the tannin levels in their diet, which would account for the lack of correlation between tannin levels and ruffed grouse feeding preference. A second explanation for the lack of correlation between tannin levels (as measured by protein precipitation) and feeding preference is that the protein-precipitating property of tannins may not be the most important biological property of tannins to assay. The soluble tannin-protein complexes, or breakdown products of tannins, and their toxic effects may be biologically more important (Vohra et al., 1966; Rogler et al., 1985; Hagerman and Robbins, 1987). Consequently, one cannot infer that the adverse effects known to be caused by a given quantity of tannic acid are equivalent to those of the tannins found in quaking aspen, even though the protein precipitating ability of the tannins may be the same.

Coniferyl benzoate has not been previously reported as occurring in quaking aspen (see Sunarjo, 1985). Earlier investigations of the secondary chemistry of quaking aspen have primarily focused on the leaves, bark, and twigs (Pearl and Darling, 1959a,b, 1964, 1965, 1967a,b, 1971; Pearl et al., 1961; Thieme, 1965, 1967; Thieme and Benecke, 1970, 1971; Wollenweber, 1975; Sunarjo, 1985). It appears as if coniferyl benzoate production is rather specialized, in that coniferyl benzoate is primarily found in flower bud scale material and not in vegetative bud scales or in other quaking aspen tissues. Other studies have shown that different tissues (e.g., buds and internodes) from the same plant not only may vary in the concentration of secondary compounds they contain, but

also may contain secondary compounds from different metabolic origins (Pearl and Darling, 1971; Mattes et al., 1987).

A comprehensive comparison between the secondary chemistry of quaking aspen flower buds and leaves was not made; however, tremuloiden, tremulacin, catechol, and isoniazid, which are present in aspen leaves (Sunarjo, 1985), were not found in flower buds.

Differences were also found in the secondary metabolite composition of the various tissues in the flower bud. As reported above, the majority of non-tannin phenolics are located in the bud scale material. Conversely, the reproductive material of the catkin contains significantly ($P < 0.001$) more protein-precipitable tannins than bud scale material. The higher ratio of reproductive to bud scale material in the catkin would account for the higher level of tannins in catkins as compared to dormant buds. It is not known how this differentiation of secondary metabolites relates to the defense of the flower bud against herbivores or plant pathogens. It may be that the phenolic compounds in the bud scales protect the bud from herbivores, such as ruffed grouse or insects, as the bud develops from late summer until spring. The higher tannin content in the reproductive tissues may be important as a defense against fungal infection as the catkin emerges from its protective bud scales in the spring and is directly exposed to environmental conditions which may promote fungal infection.

The biological properties of coniferyl benzoate have only been investigated in relation to skin allergies that are caused by various natural products used in the cosmetic industry (Hjorth, 1961; Togano et al., 1983; Kato, 1984). These studies indicate that coniferyl benzoate is one of the principle allergens found in jasmine oil and gum benzoin Siam. Coniferyl benzoate would appear to be a good alkylating agent, which would enable it to react readily with nucleophiles, including some proteins. Coniferyl benzoate may not only bind to body proteins and act as a hapten, but may also bind to dietary proteins, affecting their digestion.

Related to the allergenic properties of coniferyl benzoate is its ability to cause hypersensitivity in individuals, after initial contact with coniferyl benzoate, to coniferyl benzoate or analogous compounds (Hjorth, 1961). This property of coniferyl benzoate increases the likelihood that it may act synergistically with other secondary compounds found in quaking aspen buds. Jung and Fahey (1983) demonstrated that synergism among phenolic monomers can play an important role in depressing the food intake in rats. It is not known at this time if the other secondary compounds in aspen buds (e.g., salicin and salicortin) react synergistically with coniferyl benzoate or how they are related to ruffed grouse feeding preference. Obviously the effect that coniferyl benzoate has on ruffed grouse physiology cannot be inferred from these studies. Only by

conducting feeding trials with ruffed grouse can the feeding deterrent properties of coniferyl benzoate be confirmed and information obtained on its effect.

Feeding trials with ruffed grouse are planned that would test the hypothesis that coniferyl benzoate deters ruffed grouse from feeding on foods that have relatively high levels of this compound. Additionally, investigations are underway on how coniferyl benzoate levels are related to the differential use of quaking aspen buds by ruffed grouse during their 8- to 10-year population cycle.

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INHIBITION OF *Campoletis sonorensis* PARASITISM OF *Heliothis zea* AND OF PARASITOID DEVELOPMENT BY 2-TRIDECANONE-MEDIATED INSECT RESISTANCE OF WILD TOMATO

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Abstract—Field populations of *Heliothis* spp. were sampled for levels of naturally occurring larval parasitism on six tomato lines varying in levels of 2-tridecanone-mediated resistance to *Manduca sexta* (L.) and *Leptinotarsa decemlineata* (Say). Second and third instars were parasitized by *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) and *Cotesia* (= *Apan-*tales*) marginiventris* (Cresson) (Hymenoptera: Braconidae) in 1984 through 1986 and by *Micropletis croceipes* (Cresson) (Hymenoptera: Braconidae) in 1986. Differences in parasitism by individual and multiple species among host plants were not demonstrated. However, levels of parasitism were low and variable among replicates. Total larval parasitism averaged across all plant lines was less than 6% in 1984 and 1986 and approximately 11% in 1985. In laboratory cage studies, *C. sonorensis* parasitized fewer *H. zea* larvae on tomato foliage with high levels of 2-tridecanone than on foliage with low levels. Rearing *H. zea* on diet containing 2-tridecanone and 2-undecanone did not alter incidence of parasitism by *C. sonorensis*; nor did rearing parasitized *H. zea* larvae on chemically treated host diets precondition the parasitoid to higher or lower mortality when transferred to foliage as a substrate for cocoon spinning, regardless of the foliage genotype. However, parasitoid survival during cocoon spinning on foliage varied significantly among plant lines in a manner corresponding to the level of 2-tridecanone-mediated resistance of the foliage. Parasitoid mortality was greatest on highly resistant foliage and lowest on susceptible foliage.

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Key Words—*Camponotus sonorensis*, Hymenoptera, Ichneumonidae, *Heliothis zea*, Lepidoptera, Noctuidae, trichome, 2-tridecanone, allelochemical, plant resistance, *Lycopersicon hirsutum* f. *glabratum*, tomato.

INTRODUCTION

The resistance of the wild tomato *Lycopersicon hirsutum* f. *glabratum* C. H. Mull accession PI 134417 to the tobacco hornworm, *Manduca sexta* (L.), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is due, in large part, to the presence of a toxin, 2-tridecanone, in the tips of type VI (Luckwill, 1943) glandular trichomes, which abound on the foliage (Williams et al., 1980; Kennedy, 1986; Kennedy et al., 1987a; Fery and Kennedy, 1987). The level of resistance to both species is affected by both the amount of 2-tridecanone per trichome tip and the density of type VI glandular trichomes (Fery and Kennedy, 1987). Recently, it has been shown that the high densities of type VI trichomes found on PI 134417 foliage had a dramatic, adverse effect on parasitism of eggs of another herbivore species, *Heliothis zea* (Boddie), by *Trichogramma* spp. (Kauffman, 1987).

This study was undertaken to determine if 2-tridecanone-mediated resistance to *M. sexta* and *L. decemlineata* affected parasitoids attacking *H. zea* larvae. In addition to field experiments in which the incidence of larval parasitism by several parasitoid species was measured, we conducted detailed laboratory studies of the effects of 2-tridecanone, 2-undecanone (also a constituent of type VI trichomes on resistant foliage), and resistant foliage on the larval parasitoid *Camponotus sonorensis* (Cameron).

C. sonorensis oviposits in second- and early third-instar *H. zea* larvae. Following completion of development within the host, fifth-instar parasitoid larvae emerge through the host's integument and spin a cocoon on the foliar substrate of their host. This cocoon spinning involves extensive larval movement, which, on PI 134417, results in the destruction of large numbers of glandular trichomes.

METHODS AND MATERIALS

Larval Parasitism In Field Plots. In 1984–1986 *Heliothis* spp. larvae were collected from a 0.11- to 0.19-hectare tomato field in Clinton, North Carolina. The field was planted annually in a randomized complete block design with four to eight replicates. Individual plots were ca. 3–5 m × 1 row. Treatments consisted of the following six tomato genotypes, previously selected to provide a range of levels of 2-tridecanone-mediated resistance (Kauffman, 1987): *L. esculentum* (susceptible parental cultivar), *L. hirsutum* f. *glabratum* PI 134417

(highly resistant wild parent), F_1 (susceptible hybrid: *L. esculentum* \times PI 134417), and three backcross ($F_1 \times$ PI 134417) accessions BC17, BC2, and BC90, classified as having low, moderate, and high levels of 2-tridecanone-mediated resistance, respectively. The backcross lines were propagated vegetatively from individual plants selected from an F_1 backcross population. Each year the level of tobacco hornworm resistance of field-grown plants was verified by bioassay and gas chromatographic (GC) analysis of type VI trichome exudate to quantify 2-tridecanone and 2-undecanone levels. 2-Undecanone concentrations were of interest because, at concentrations associated with PI 134417 foliage, it causes pupal mortality of *H. zea* when ingested during the last larval instar. It has also been shown to synergistically enhance toxicity of 2-tridecanone to several species (Farrar and Kennedy, 1987a; Lin et al., 1987) but has no apparent effect on tobacco hornworm.

The hornworm bioassay consisted of two *M. sexta* neonates placed on the abaxial surface of a 78.5-mm² leaflet disk excised from the uppermost fully expanded leaf. The leaf disk was placed on moistened filter paper in a 5-cm plastic Petri dish, which was then sealed with Parafilm and held at 27°C. For each plant, this procedure was replicated with 6–10 disks obtained from separate leaflets. After 24 hr, consumption was measured to the nearest square millimeter by placing the leaf disk remnants on a 78.5-mm² circular grid divided in 1-mm² units and tallying the missing foliage. Walter and PI 134417 were included as susceptible and resistant controls, respectively. Type VI trichome density was determined by counting all type VI trichomes present on each of two to three 28.3-mm² leaflet disks taken from the uppermost fully expanded leaf of each plant. Concurrently, 1200 type VI trichome tips were collected from test foliage, using glass microcapillary tubes. Tip extracts were dissolved in HPLC-grade methanol and analyzed on a Hewlett-Packard 5880A gas chromatograph, using a Durabond 15-m \times 0.25- μ m fused silica capillary column with an injection volume of 10 μ l/sample and temperature program of 100–275°C increasing at 5°C/min.

The natural incidence of larval parasitism was determined by collecting larvae with a 60 \times 60-cm, beat cloth approximately 10 days after the ovipositional peak of second generation moths. *Heliothis* spp. larvae were categorized by instars, then placed individually into 30-ml plastic diet cups containing a corn-soya-milk (CSM) diet (Burton, 1970). Larvae were held at 27°C and 16:8 (light-dark) photoperiod and monitored daily for evidence of parasitism (i.e., cessation of growth at ca. 15 mm or less). Emerged adult parasitoids were identified to species. Host larvae collected from the same plant line were pooled prior to determination of parasitism. Percent parasitism (by parasitoid species and total parasitism) was analyzed by chi square for significant differences ($P \leq 0.05$) among plant lines.

Laboratory Studies. The levels of 2-tridecanone-based resistance and the

concentrations of 2-tridecanone and 2-undecanone were determined for all greenhouse-grown plants used in these studies by means of the aforementioned tobacco hornworm bioassay and GC analysis of trichome tips. The parasitoids came from a culture of *C. sonorensis* maintained on *H. zea* in the laboratory under conditions of 27°C and a light-dark cycle of 16:8. *H. zea* larval hosts were obtained from a laboratory colony maintained on CSM diet at North Carolina State University.

To test the hypothesis that, under constant conditions of host size and density, adult *C. sonorensis* would utilize all tomato lines equally well as substrates for parasitism of *H. zea*, we exposed mated adult female *C. sonorensis* to equal densities of *H. zea* larvae on foliage of each of the following plant lines in a no-choice experiment: *L. esculentum*, BC17, BC2, BC90, and PI 134417. Foliage from greenhouse-grown plants was arranged in bouquets consisting of five young fully expanded leaves with their petioles in a water-filled beaker. One bouquet (hence one plant line) was placed in each 30 × 30 × 30-cm Saran screen cage. Thirty second-instar, diet-reared *H. zea* larvae were placed on each bouquet and three 4-day-old, mated, adult, female *C. sonorensis* added to each cage. The infested cages were held at 27°C for 8 hr, after which the *H. zea* larvae were transferred to and maintained individually on CSM diet. Ten days later, larvae were classified as live and unparasitized, dead, or parasitized. Adult emergence and sex of *C. sonorensis* were recorded on day 20 for all parasitized hosts. Each plant line was replicated 20 times.

Data from this and all subsequent experiments were subjected to analysis of variance with mean separation using Duncan's new multiple-range test. All percentage data were based on numbers of larvae accounted for at the termination of the test and were transformed to arcsine $\sqrt{\text{percentage}}$ prior to analysis.

To determine if the presence of 2-tridecanone and 2-undecanone in the host's diet affected parasitism by *C. sonorensis* and if tomato foliage differing in levels of 2-tridecanone-mediated resistance to *M. sexta* differed in suitability as a pupation substrate for *C. sonorensis*, the following experiment was conducted. *H. zea* larvae were reared on three diets: (1) standard CSM diet (Burton, 1970), control; (2) CSM diet containing 0.3% and 0.055% (w/w) 2-tridecanone and 2-undecanone, respectively, concentrations which approximate those found in PI 134417 foliage; or (3) CSM diet containing 0.075% and 0.0137% (w/w) 2-tridecanone and 2-undecanone, respectively, concentrations approximating those found in BC2 foliage. The 2-tridecanone (Fluka Chemical Corp., Haulpauge, New York 11788) and 2-undecanone (Pfaltz and Bauer, Inc., Stamford, Connecticut 06902) were greater than 99% pure as determined by gas chromatography. Chemically treated diets were prepared as described by Farrar and Kennedy (1987).

H. zea larvae were reared to late second instar on the various diets, sub-

jected to parasitism by *C. sonorensis*, and returned to their respective diets and maintained at 27°C. Ten days later the larvae were classified as live and unparasitized, dead, or parasitized. Larvae were exposed to parasitism by confining them for 8 hr in groups of 10 in an inverted plastic cup (Sweetheart Plastics, Wilmington, Massachusetts) (9 cm diam. × 4.5 cm high) containing three cubes of CSM diet and one mated adult *C. sonorensis* female. Data were collected for 1002 larvae on the control diet and 985 and 823 larvae on the diets containing low and high concentrations of the test chemicals, respectively. Shortly before parasitoid larvae emerged from their hosts, indicated by pink coloration of the posterior third of host larva, the parasitized larvae from each diet were transferred in groups of five to the abaxial surface of excised foliage of *L. esculentum*, PI 134417, or BC2. This foliage was maintained at 27°C on moist filter paper in Petri dishes sealed with Parafilm. Twenty-one days following exposure of host larvae to adult *C. sonorensis*, the experiment was terminated and parasitized larvae were classified as producing a *C. sonorensis* larva that died prior to initiation of cocoon construction, a *C. sonorensis* larva that died during cocoon construction, or a complete cocoon. There were 15 replicates of 10 parasitized hosts for each diet-foilage treatment.

RESULTS

Larval Parasitism In Field Plots. Levels of resistance of field grown-plants varied somewhat from year to year, as indicated by hornworm foliar consumption and GC analysis of 2-tridecanone concentrations in trichome exudate. However, *L. esculentum* and F₁ were consistently highly susceptible, whereas BC90 and PI 134417 were consistently highly resistant (Table 1). Similar patterns of resistance and methyl ketone concentrations occurred in the glasshouse-grown plants used for laboratory studies with *C. sonorensis* (Table 2).

Second and third instars of *Heliothis* spp., which were the only stadia successfully parasitized, represented 77% ($N = 774$), 43% ($N = 325$), and 67% ($N = 791$) of the total larvae collected in 1984, 1985, and 1986, respectively. They were parasitized by *C. sonorensis* and *Cotesia marginiventris* (Cameron) in all three years and by *Microplitis croceipes* (Cresson) only in 1986 (Figure 1). Neither levels of parasitism by individual species nor total parasitism were significantly affected by tomato line (chi square, $P \leq 0.05$). Total parasitism averaged over all plant lines was less than 6% in 1984 and 1986 and ca. 11% in 1985. Data from 1985 was based on a relatively small number of hosts due to low *H. zea* oviposition (Kauffman, 1987). Also, the fewest *H. zea* larvae were collected on *L. esculentum* due to lower oviposition and higher egg parasitism by *Trichogramma* spp. than on the other plant lines (Kauffman, 1987).

Parasitism in Cages. Fewer *H. zea* larvae were parasitized by *C. sonorensis*

TABLE 1. LEVELS OF RESISTANCE-ASSOCIATED PARAMETERS IN FIELD-GROWN TOMATO PLANTS (CLINTON, NORTH CAROLINA, 1984-1986)^a

Year	Plant line	Trichome density (No./mm ²)	Conc. 2-tridecanone (ng/mm ²)	Conc. 2-undecanone (ng/mm ²)	Hornworm consumption (%)
1984 ^b	<i>L. esculentum</i>				61.0 ab
	F1				68.8 a
	BC17	13.0 b	5.8 b	0 b	73.3 a
	BC2	10.5 b	5.9 b	0 b	50.2 b
	BC90	19.0 a	131.6 a	36.8 a	21.7 c
	PI 134417	21.7 a	86.0 a	19.9 a	14.6 c
1985 ^c	<i>L. esculentum</i>				65.9 ab
	F1				81.7 a
	BC17	13.8 a	15.9 b	12.2 b	59.5 b
	BC2	17.1 a	57.3 b	10.6 b	32.2 c
	BC90	15.7 a	233.1 a	56.8 a	12.7 d
	PI 134417	17.8 a	183.2 a	44.3 a	10.7 d
1986 ^d	<i>L. esculentum</i>	1.2 d	0 c	0 c	91.5 a
	F1	10.2 c	0 c	0 c	87.3 a
	BC17	15.0 b	17.8 b	2.4 b	86.4 a
	BC2	15.2 b	80.2 b	18.8 b	77.7 a
	BC90	20.6 a	318.2 a	87.8 a	12.3 b
	PI 134417	23.3 a	352.8 a	96.3 a	16.6 b

^aVertical separation of means within years at $P \leq 0.05$ by Duncan's new multiple-range test.

^bSampled on July 31; four replications per plant line; 300 trichome tips collected per sample for gas chromatography.

^cSampled August 4; eight replications; 1200 tips collected/sample.

^dSampled August 16; six replications; 1200 tips collected/sample.

TABLE 2. LEVELS OF RESISTANCE OF GLASSHOUSE-GROWN PLANTS USED IN LABORATORY STUDIES WITH *C. sonorensis*, 1985.

Plant line	Mean \pm SD			
	Trichome density ^a (No./mm ²)	Conc. 2-tridecanone ^b (ng/mm ²)	Conc. 2-undecanone ^b (ng/mm ²)	Hornworm consumption ^c (%)
<i>L. esculentum</i>				91.1 (10.21)
BC17	17.3 (4.63)	10.1 (0.70)	0	87.7 (13.42)
BC2	20.1 (2.95)	30.7 (8.20)	4.2 (1.40)	60.9 (24.94)
BC90	21.2 (1.18)	201.0 (81.43)	39.2 (17.78)	1.7 (1.74)
PI 134417	15.2 (0.43)	165.4 (43.70)	27.4 (1.76)	5.9 (6.12)

^aTwo leaflet disks/replicate.

^bBased on analysis of composite samples of 1200 trichome tips/replicate.

^cSix replications.

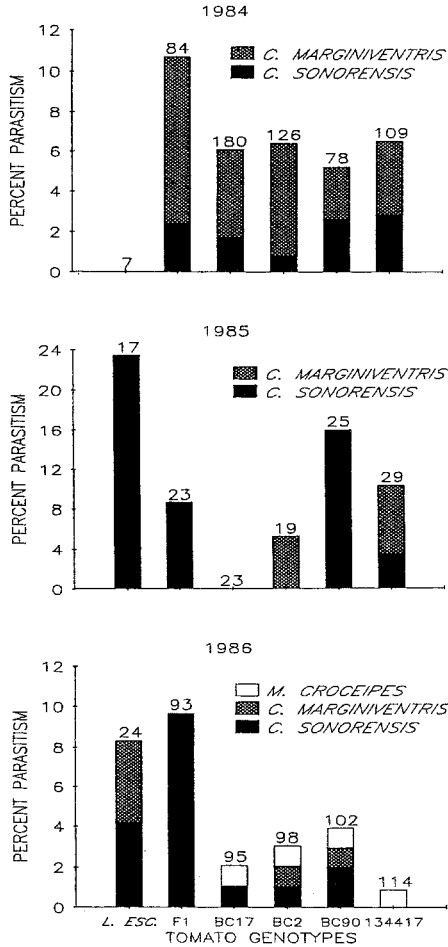


FIG. 1. Field parasitism of *Heliothis* spp. second and third instars in tomato; Clinton, North Carolina. Numbers above the vertical bars indicate the total number of larvae collected.

sis in laboratory cages on highly resistant (PI 134417 and BC90) plants than on BC2, BC17, or *L. esculentum* plants (Table 3). The percentage of dead host larvae was unaffected by plant line, suggesting that contact with and ingestion of resistant foliage by *H. zea* larvae during the brief period of exposure to adult parasitoids had a negligible influence on their survival. Furthermore, such exposure did not result in increased premature mortality of parasitized hosts, nor did it alter emergence success or sex ratio of adult parasitoids from parasitized hosts (Table 4). Since confinement of adult parasitoids in the cage with foliage did

TABLE 3. OUTCOME OF EXPOSING *H. zea* LARVAE TO *C. sonorensis* IN CAGES^a

Plant line	Level of resistance	Percent \pm SD		
		Dead hosts	Live, unparasitized	Parasitized
<i>L. esculentum</i>	Susceptible	4.8	30.5 a	64.6 a
BC17	Low	4.0	30.8 a	65.2 a
BC2	Moderate	5.5	40.4 a	54.1 a
BC90	High	6.3	62.7 b	31.0 b
PI 134417	High	4.5	56.6 b	39.0 b
		NS		

^aTwenty replications of cages per treatment; data transformed by arcsine $\sqrt{\text{percentage}}$ prior to ANOVA and vertical separation of means ($P \leq 0.05$).

not result in death of the parasitoids, it is possible that the adults were deterred from prolonged close-range aerial or tactile examination of resistant foliage for the presence of acceptable hosts, thus reducing parasitism.

The laboratory data appear to disagree with field parasitism data (Figure 1), which indicated no effect of plant genotype on parasitism by *C. sonorensis*. This inconsistency probably is due to the low and highly variable rates of parasitism observed in the field studies, but possible effects of confinement in relatively small cages cannot be discounted. Further field studies are warranted.

Effects of Host Diet and Pupation Substrate on C. sonorensis. Parasitism of *H. zea* larvae reared on diet containing 2-tridecanone and 2-undecanone at concentrations approximating those found in foliage of BC2 (low) and PI 134417 (high) plants did not differ from that on the control diet (Figure 2). Following

TABLE 4. *C. sonorensis* PROGENY RESULTING FROM PARASITISM OF *H. zea* IN CAGES^a

Plant line	Total No. parasitized	Cocoons with emerged parasitoid adults, % (\pm SD)	Female progeny, % (\pm SD)
<i>L. esculentum</i>	493	93.9 (8.33)	47.0 (45.55)
BC17	499	95.4 (6.33)	50.6 (45.94)
BC4	463	91.6 (9.41)	49.4 (48.88)
BC90	150	93.4 (11.03)	40.1 (30.50)
PI 134417	183	91.8 (9.98)	43.0 (49.96)

^aTwenty replications; arcsine $\sqrt{\text{percentage}}$ transformation, ANOVA ($P \leq 0.05$, DNMR). Treatment means not significantly different at $P \leq 0.05$.

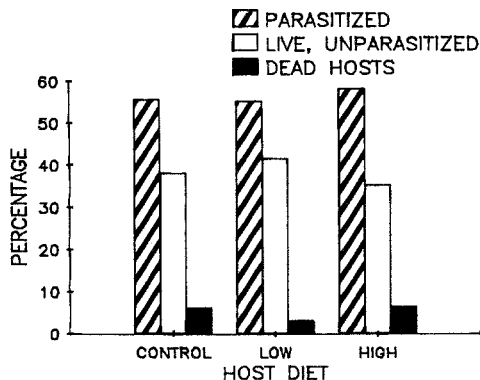


FIG. 2. Outcome of exposing *H. zea* larvae reared on chemically treated and untreated diets to *C. sonorensis*. Low and high refer to concentrations of 2-tridecanone and 2-undecanone in the diets. See text for details.

H. zea exposure to *C. sonorensis*, there were no differences in the percentages of dead hosts and live, unparasitized hosts due to diet consumed by *H. zea*.

The concentration of 2-tridecanone in the diet of the host larvae did not significantly alter survival of *C. sonorensis* either during larval development within the host or subsequently during cocoon spinning and pupal development. Thus, for any given plant line on which pupation occurred, there were no significant differences among host diets in percent parasitoid mortality (Table 5). The foliage type on which cocoon spinning and pupation occurred, however, had a profound effect on *C. sonorensis* larval and pupal mortality; this effect was independent of the 2-tridecanone concentration of the host's diet (Table 5). Parasitoid mortality during and after cocoon spinning closely paralleled the 2-tridecanone and 2-undecanone concentrations of the foliage that served as a substrate for cocoon spinning. Mortality was least on *L. esculentum*, which contained only trace quantities of 2-tridecanone and 2-undecanone, intermediate on BC2 foliage, which contained a mean of 31 ng 2-tridecanone and 4 ng 2-undecanone per mm², and greatest on PI 134417 foliage, which averaged 165 ng 2-tridecanone and 27 ng 2-undecanone per mm² (Tables 4 and 5).

DISCUSSION

Tomato genotypes possessing high levels of 4-tridecanone and 4-undecanone resulted in decreased parasitism of *H. zea* by *C. sonorensis* and interfered with successful cocoon spinning by the parasitoid in laboratory studies. During cocoon construction on the foliage of resistant plants, fifth-instar *C. sonorensis* disrupt numerous type VI glandular trichomes and directly contact the 4-tri-

TABLE 5. MORTALITY OF *C. sonorensis* WHEN PLACED ON FOLIAGE FOR COCOON SPINNING FOLLOWING DEVELOPMENT OF PARASITIDS IN *H. zea* LARVAE REARED ON CHEMICALLY TREATED AND UNTREATED DIETS^a

Foliage for cocoon spinning	Larval diet of host ^b	Dead parasitoids (%)		
		Larvae ^c	Pupae and unemerged adults	All stages ^d
<i>L. esculentum</i>	Control	0.7 a	9.5 a	10.2 a
	Low conc.	3.5 a	5.8 a	9.3 a
	High conc.	3.4 a	9.0 a	12.4 a
BC2	Control	43.6 c	16.3 b	59.9 b
	Low conc.	28.7 b	16.3 b	45.0 b
	High conc.	51.7 c	12.5 b	64.2 b
PI 134417	Control	98.0 d	0.7 c	98.7 c
	Low conc.	94.0 d	0.0 c	94.0 c
	High conc.	97.3 d	0.0 c	97.3 c

^aFifteen replications; arcsine $\sqrt{\text{percentage}}$, ANOVA ($P \leq 0.05$, DNMR7).

^bLow conc. = 0.075% 2-tridecanone + 0.0137% 2-undecanone (w/w); high conc. = 0.3% 2-tridecanone + 0.055% 2-undecanone (w/w).

^cData include all larvae that died prior to completing cocoon construction.

^dData include larvae, pupae, and unemerged adults.

decanone and 4-undecanone contained in their tips. Since 4-tridecanone is highly toxic to *C. sonorensis* larvae (Kauffman, 1987), it likely accounts for the observed mortality on resistant foliage during cocoon spinning. It is noteworthy that on foliage containing high levels of 4-tridecanone (PI 134417), virtually all parasitoid mortality occurred during cocoon spinning, whereas on foliage with intermediate levels of 4-tridecanone (BC4), a significant portion of the total parasitoid mortality occurred subsequently among pupae and unemerged adults. This may be attributable to the reduced dosages experienced on BC4 foliage. When incorporated into the host's diet, these two methyl ketones had no observable effect on the suitability of the host larvae for parasitism by adult *C. sonorensis* or for parasitoid development within the host. In the latter situation, the parasitoid larvae would contact the chemicals only if they were circulated or deposited within their host.

Selection of tomato lines for high levels of foliar 2-tridecanone could result in valuable resistance to both tobacco hornworm and Colorado potato beetle. In some situations, however, the advantages of this resistance may be more than offset by adverse effects on parasitism of *H. zea* eggs by *Trichogramma* spp. (peak percentage parasitism > 90% on *L. esculentum* vs. < 20% on highly resistant lines; Kauffman, 1987), and on larval parasitism by *C. sonorensis*, as

well as by the induction of elevated levels of cytochrome P-450 isozymes and associated elevated levels of tolerance to some insecticides in *H. zea*, which follows exposure to 2-tridecanone or resistant foliage (Kennedy, 1984; Kennedy et al., 1987b). Clearly, where *H. zea* is a potentially serious pest on tomato, it would be inappropriate to use tomato cultivars selected for high levels of 2-tridecanone-mediated resistance to other insect pests, unless those cultivars also possessed substantial levels of *H. zea* resistance attributable to other factors.

The ecological and practical consequences of the effects of 2-tridecanone-mediated resistance on parasitism by *C. sonorensis* will depend on the role of the parasitoid in suppression of *H. zea* populations on tomato. In the Coastal Plain of North Carolina, *C. sonorensis* seems to be of relatively minor importance in the population dynamics of *H. zea* on tomato, but in other areas it may play a much more important role.

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ISOLATION AND IDENTIFICATION OF MALE MEDFLY ATTRACTIVE COMPONENTS IN *Litchi chinensis* STEMS AND *Ficus* SPP. STEM EXUDATES¹

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Abstract—Short-range attraction/feeding stimulation of male Mediterranean fruit flies [*Ceratitis capitata* (Wiedemann), (Diptera: Tephritidae)] to a stem extract of a host plant, *Litchi chinensis* Sonn. (Sapindaceae), and to milky exudates from stems of nonhost plants, *Ficus retusa* L. and *F. benjamina* L. (Moraceae), were attributed to the presence of the sesquiterpene α -copaene. The presence of α -copaene in the milky exudate from stems of *F. benghalensis* L. is also suggested as eliciting similar behavioral responses in male medflies. The presence of minor quantities of α -ylangene in the plants and its contributory effects to the behavioral response of male medflies is discussed. Short-range attraction/feeding stimulation of male medflies to equal amounts of α -ylangene-free α -copaene samples (94.5%+), prepared from α -copaene-enriched angelica seed oil and copaiba oil, respectively, showed no difference in intensity of response. α -Ylangene elicited a slightly less intense response for male medflies than α -copaene.

Key Words—*Litchi chinensis*, Sapindaceae, *Ficus retusa*, *Ficus benjamina*, *Ficus benghalensis*, Moraceae, *Ceratitis capitata*, Diptera, Tephritidae, Mediterranean fruit fly, medfly, α -copaene, α -ylangene.

¹Names of products in this paper are included for the benefit of the reader and do not imply endorsement or preferential treatment by USDA.

INTRODUCTION

Naturally occurring attractants for *Ceratitis capitata* (Wiedemann) have been found in extracts and oils of many plants (Beroza and Green, 1963a). These include extracts of *Alpinia officinarum*, *Anaphalia margaritacea*, *Angelica* spp., *Berberis vulgaris*, *Chimaphila umbellata*, *Conioselinum chinense*, *Dendrobium superbum*, *Equisetum arvense*, *Fabiana imbricata*, *Festuca* spp., *Heracleum lanatum*, *Juglans nigra*, *Lycopersicon esculentum*, *Rhamnus frangula*, *Rosa centifolia*, *Tilia europaea*, and oils (Teranishi et al., 1987) of *Abies alba*, angelica seed (Steiner et al., 1957; Beroza and Green, 1963b; Fornasiero et al., 1969; Jacobson et al., 1987), copaiba, cubeb, cyste, gingergrass, grapefruit, hop, lemon, orange, Canadian pine needle, *Pinus pumilio*, *P. sylvestris*, sweet orange, orange peel, and ylang-ylang.

The present study extends this list of naturally occurring attractants for *C. capitata* to the host plant, *Litchi chinensis* Sonn. (Sapindaceae) (Back and Pemberton, 1918a,b), *Ficus benghalensis* L. (Moraceae)/*F. indica*, which has conflicting reports of host vs. nonhost classification (Quayle, 1938; Back and Pemberton, 1918a,b), and several nonhost *Ficus* spp.

L. chinensis, litchi or lychee, is a medium sized-tree that is native to the humid regions of southern China, where it has grown for several thousand years. Thousands of tons of the dried fruit are exported for use by Chinese people living abroad. These fruits are known as Chinese litchi nuts in the Western world. The trees are also cultivated in the American subtropics (Dahlgren, 1947).

McInnis and Warthen (1988) observed that the exudate from a stem of *F. benjamina* L., weeping fig, attracted male medflies and stimulated them to feed. Other species of *Ficus*, namely *F. benghalensis* L. (Indian banyan) and *F. retusa* L. (Indian laurel fig), produced the same behavioral responses (Table 1). We did not investigate *F. carica*, the common fig, which is a host for medfly. This plant is listed as being heavily or generally infested by medflies (Back and Pemberton, 1918a,b).

A detailed procedure was developed to isolate and identify the short-range attractants/feeding stimulants from *L. chinensis*; this same procedure was also applied to the *Ficus* spp. for isolation and identification.

METHODS AND MATERIALS

L. chinensis stems were collected and dried at two locations: the Tropical Agriculture Research Station, Mayaguez, Puerto Rico, and at the Subtropical Horticulture Research Laboratory, Miami, Florida. Leaves, small stems, and twigs of *F. retusa* and *F. benjamina* were also obtained and dried at Mayaguez.

TABLE 1. SHORT-RANGE LABORATORY MEDFLY ATTRACTANCY/FEEDING STIMULATION SCORING SYSTEM^a

Qualitative score	Quantitative score ^b
Negative (-)	No reaction above normal "passerby" activity.
Slight to negative (sl to -)	0-5 flies feeding or "sitting" on blotter within 5 min of start.
Slight (sl)	5-10 flies feeding, "mating," ^c or resting on blotter within 5 min.
+	10-20 flies feeding, etc., on blotter, within 2 min.
++	20-40 flies feeding, etc., on blotter within 2 min. Male "mating" activity heavy.
+++	40-60 flies feeding, etc., within 2 min. Male "mating" activity heavy.

^aMcInnis and Warthen, 1988.

^bApproximately 200 flies/sex for each test.

^c"Mating" by males consisted of attempted copulations by two or more individuals mounted in a row.

Leaf stem exudates were obtained from *F. benjamina* at the USDA Tropical Fruit and Vegetable Laboratory, Honolulu, Hawaii; from *F. retusa* on the east side of Oahu, Hawaii; and from *F. benghalensis* at the U.S. Botanic Gardens, Washington, D.C. A small sample of *F. benghalensis* fresh leaves was also obtained from the latter source.

Solvents and Sesquiterpenes. *n*-Hexane and isooctane were HPLC grade; all other solvents were reagent grade. The sources of sesquiterpenes, including enriched α -copaene samples from angelica seed oil and copaiba root oil, are listed in Table 2.

Extractions and Essential Oils. Dried stems (231.3 g) of Puerto Rican *L. chinensis* were ground in a Wiley mill and extracted with *n*-hexane for 24 hr in a Soxhlet apparatus; the marc was extracted with ethyl ether for 24 hr. Similar extractions of the dried stems of Miami *L. chinensis* were also performed. Fresh leaves (91.0 g) of *F. benghalensis* were ground in a Waring blender with *n*-hexane and extracted for 18 hr with *n*-hexane in a Soxhlet apparatus; the marc was extracted with ethyl ether for 18 hr. Dried leaves, small stems, and twigs (307.3 g) of *F. retusa* were ground in a Wiley mill and extracted with ethyl ether for 72 hr in a Soxhlet apparatus. Similar extractions of the dried leaves, small stems, and twigs (323.9 g) of *F. benjamina* were also performed. A steam distillate (collected in *n*-hexane, utilizing a continuous extraction head for 24 hr) was prepared from each of the following: 64.3 g ethyl ether extract of Puerto Rican *L. chinensis* (1498 g), 26.1 g ethyl ether extract of Miami *L. chinensis* (804 g), 48.9 g ethyl ether extract of *F. retusa* (307.3 g), and 40.6 g ethyl ether extract of *F. benjamina* (323.9 g).

TABLE 2. GLC AND HPLC RETENTION TIMES OF STANDARD SESQUITERPENES

Sesquiterpene	Source	GLC Retention time ^a (min)	HPLC Retention time ^b (min)
(+)- α -Longipinene	Fluka	8.79	20.93
(+)-Longicyclene	Fluka	9.03	
α -Ylangene	^c	9.16	27.06
α -Copaene	^d	9.24	29.33
(-)-Isolongifolene	Fluka	9.29	
(+)-Longifolene	Aldrich	9.64	22.06
α -Cedrene	Aldrich	9.76	12.60
(-)- β -Caryophyllene	Fluka	9.92	^e
(-)-Thujopsene	Fluka	10.06	32.60 ^f
(+)-Calarene	Fluka	10.13	13.46
(β -Gurjunene)			
(+)-Aromadendrene	Fluka	10.29	
α -Humulene	Fluka	10.59	
(α -Caryophyllene)			
Alloaromadendrene	Fluka	10.69	
β -Gurjunene	Fluka	11.07	
Eremophilene	Roth	11.07	
(labeled as valencene)			

^aRetention times are for elution comparison within the table and are not exactly superimposable upon retention times in Figure 2.

^bSome tailing of the monounsaturated sesquiterpenes occurs with these analyses.

^cObtained from Professor V. Herout, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Prague.

^dObtained from Professor Buchi, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts. Also obtained from M. Jacobson; USDA-Retired, Collaborator (Jacobson et al., 1987).

^e(-)-Thujopsene and (-)- β -caryophyllene were separated on 3 μ m silica under the same HPLC conditions, giving 9.46 and 11.26 min retention times, respectively.

Each of the above extracts were dried (sodium sulfate) and concentrated in vacuo. The steam distillates were concentrated to 5.0 ml solutions.

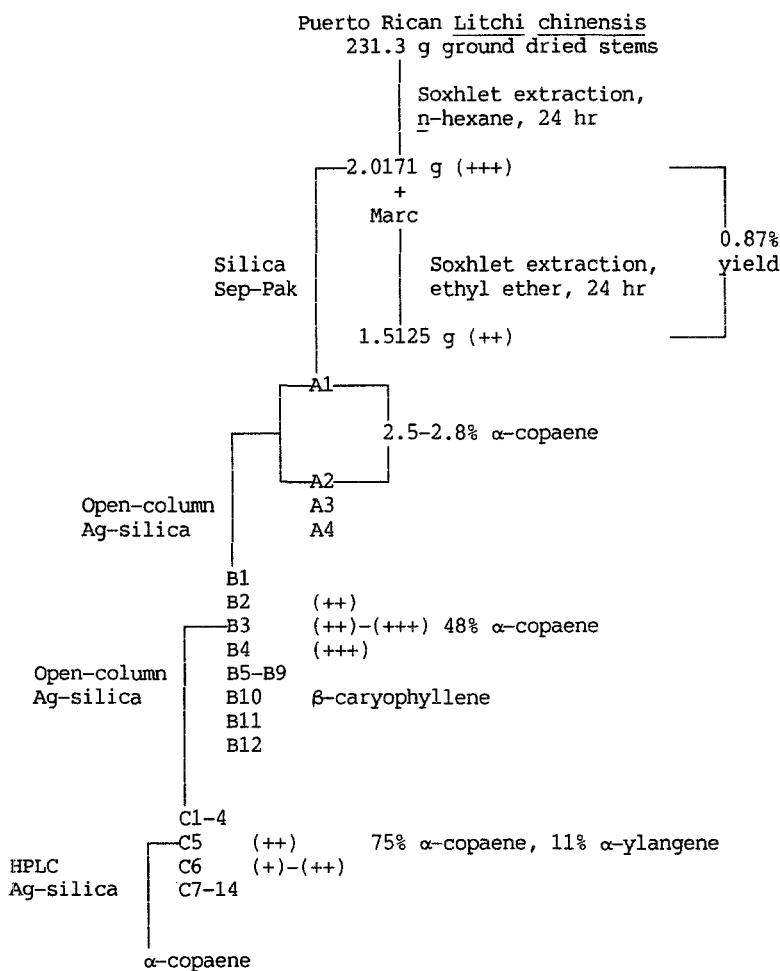
Exudates. Exudates of *F. benjamina* and *F. benghalensis* were collected with Pasteur pipets from snapped leaf stems. The exudates were refrigerated as such or mixed with distilled water (2 drops exudate/1.0 ml water) and refrigerated. Two of the latter *F. benghalensis* mixtures were extracted with 1.0 ml *n*-hexane and 1.0 ml ethyl acetate, respectively.

Leaf stem exudates of *F. retusa* were similarly collected, and trunk exudates were collected through a wound made in the trunk. These exudates were extracted with an equivalent volume of methylene chloride; the organic layer was stored under refrigeration.

Open-Column Chromatography. Millipore Waters Associates Sep-Pak sil-

ica cartridges were prewashed with 5.0 ml methylene chloride and then 10.0 ml *n*-hexane. The *n*-hexane extract (1–5 drops from a Pasteur pipet) of *L. chinensis* was placed on the Sep-Pak and eluted with three 5.0-ml portions of *n*-hexane followed by 5.0 ml methylene chloride; four 5.0-ml fractions (A1–4, Scheme 1) were collected.

To 437 g Bio-Rad Bio-Sil A (100–200 mesh) was added an aqueous, saturated solution of 109.25 g reagent-grade silver nitrate with shaking; the mixture was heated at 125°C overnight. The first two fractions, A1 and A2, were



SCHEME 1. See Table 1 for qualitative scoring (+, ++, +++) for short-range laboratory medfly attractant/feeding stimulation.

placed on a column (3.0 × 0.5 cm ID) containing 0.32 g of this adsorbent (20% silver nitrate) that was first wetted and prewashed with 2.0 ml *n*-hexane. Portions (0.7 ml each) of *n*-hexane, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 8.0, 16.0, 32.0, 64.0% ethyl ether/*n*-hexane, and ethyl ether were added to the column and twelve 0.7-ml fractions (B1–12) were collected.

B3, concentrated to a 50- μ l volume, was placed on another prewashed silver nitrate–Bio-Sil A column of the same configuration and eluted with portions (0.7 ml each) of *n*-hexane, 0.5, 1.0, 1.5, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10.0, 20.0, and 40.0% ethyl ether/*n*-hexane; each of the fourteen 0.7-ml fractions (C1–14) was concentrated.

A 4.1-ml methylene chloride extract of 4.0 ml *F. retusa* exudate was concentrated under nitrogen to 0.6 ml; three 0.2-ml aliquots of this concentrate were each placed on a prewashed silica Sep-Pak and eluted with *n*-hexane as previously described. The first fractions from each Sep-Pak were combined and concentrated under nitrogen to 0.2 ml. An aliquot (150 μ l) of this concentrate was placed on a 20% silver nitrate–Bio-Sil A column as previously described and eluted with portions (0.7 ml each) of *n*-hexane, 1.0, 2.0, 4.0, 8.0, 12.0, 18.0, 27.0, 40.0, 60.0% ethyl ether/*n*-hexane, and ethyl ether.

High-Performance Liquid Chromatography (HPLC). Analytical and preparative HPLC were carried out on a Waters Associates ALC-100 equipped with a model 720 system controller, a model 730 data module, two model 6000A pumps, a U6K injector, and a model 440 absorbance detector with an extended wavelength module at 214 nm.

Silver nitrate (20% w/w) on 3 μ m Hypersil (Shandon) was prepared by adding a solution of 2.5 g reagent grade silver nitrate in 70 ml acetonitrile to a mixture of 10 g 3 μ m Hypersil in acetonitrile (Heath et al., 1975). The mixture was concentrated in vacuo to dryness, and 4.0 g was added to 30 ml carbon tetrachloride and sonicated for several minutes. The mixture was added to a stainless-steel reservoir and topped off with carbon tetrachloride (Heath et al., 1977). The mixture was pumped into a 30 × 0.39 cm ID stainless-steel column followed by 50 ml *n*-hexane at 9000 psi air pressure. Then, the packed column was flushed with 5% ethyl ether–isooctane at 1.5 ml/min, until 400 ml was collected, and further flushed with isooctane until all traces of ethyl ether were removed.

Sesquiterpenes (50–100 μ g) in a volume of 5–100 μ l *n*-hexane solution were injected for analysis at a flow rate of 0.5 ml/min isooctane. After maintaining this flow rate for 15 min, a linear flow program was then initiated over 5 min to 1.0 ml/min isooctane followed by a linear gradient program over the next 10 min to 0.5% ethyl ether–isooctane. C5, containing about 12 μ g α -copaene in a 78- μ l volume of solvent, was also injected; α -copaene was collected and concentrated under nitrogen to a 100- μ l volume.

GLC. A Hewlett-Packard 5880A gas chromatograph equipped with a cap-

illary injector system and a flame ionization detector was used for GLC analyses. A Hewlett-Packard 12 m \times 0.2 mm ID dimethyl silicone (DMS) fused-silica capillary column was used for the analyses.

Temperatures employed for GLC were: injection port 200°C; detector 220°C; DMS column, 50°C for 5 min, then programmed at 20°C/min to 105°C, maintained at 105°C for 7.25 min, then programmed at 10°C/min to 125°C. Total run time was 22 min. A split ratio of 1:188 was used with a helium flow of 2.2 cm³/min plus an appropriate amount of helium makeup gas.

Standard sesquiterpene samples were dissolved in *n*-hexane to make 0.1% solutions, and 1 μ l of the solutions were injected for GLC analyses. The concentration of unknowns in solution for GLC analyses was adjusted to match that of standards in most cases.

Gas Chromatography-Mass Spectrometry (GC-MS). Mass spectra were recorded on a Finnigan GC-MS 4500 with a 6000 data system. The GC component was equipped with a 60 m \times 0.25 mm ID DB-1 capillary column (J&W Scientific) for sesquiterpene analyses in Figures 1A and 1B and a 50 m \times 0.25 mm ID Ultra 1 (100% dimethyl polysiloxane gum) capillary column (Hewlett-Packard) for analyses of α -copaene in Figures 1C and 1D. In the EI mode, 70 eV energy was used.

Bioassay. A rapid and efficient short-range laboratory bioassay was developed (McInnis and Warthen, 1988) to evaluate crude and fractionated plant extracts, purified phytochemicals, and standard sesquiterpenes as attractants/feeding stimulants for medflies, *C. capitata*. The bioassay's qualitative scoring system with a quantitative and behavioral basis appears in Table 1. Typically, 1- μ g samples of purified phytochemicals and standard sesquiterpenes were utilized. The concentrations of crude and fractionated plant extracts were adjusted according to the percentage of active substance present.

RESULTS

A Soxhlet extraction (2.5–5.1% yield) of Puerto Rican *L. chinensis* (Scheme 1) was also obtained with ethyl ether for 72–96 hr (the extract obtained during the last 24 hr gave a rapid +++ in the bioassay); extraction over 96 hr

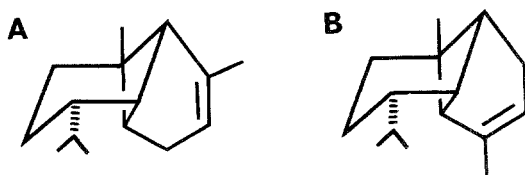


FIG. 1. Structural formulae of (-)- α -copaene (A) and (+)- α -ylangene (B).

with *n*-hexane alone gave only a 1.7% yield with a slow +++ in the bioassay. The Miami *L. chinensis*, when extracted with ethyl ether in a Soxhlet for 72 hr, gave a yield ranging from 2.6 to 5.9% that elicited less biological activity than the Puerto Rican variety.

Biologically active A1 and A2 (Scheme 1) from the Puerto Rican *L. chinensis* *n*-hexane extract showed the possible presence of α -ylangene (Figure 1B), α -copaene (2.5–2.8%, Figure 1A), β -caryophyllene, α -humulene, longifolene, and eremophilene by retention time comparison with standard sesquiterpenes (Table 2) via GLC analyses. Chromatography of A1 and A2 revealed biological activity centered in B3, which appeared to contain α -copaene (48%) by GLC analyses. The presence of β -caryophyllene (95%) in B10 was confirmed by GC-MS with an NIH/EPA Spectral Library match. This substance was isolated and identified since it was listed as an insect attractant for medflies and Oriental fruit flies (Beroza and Green, 1963a). However, a Fluka β -caryophyllene sample (99.7% by GLC analysis) indicated no biological activity for medflies or Oriental fruit flies. K&K and Givaudan β -caryophyllene samples that were attractive to male medflies revealed the presence of 0.38–0.57% and 0.41% α -copaene, respectively, by GLC retention time comparison with standard α -copaene.

Chromatography of B3 revealed biological activity centered in C5, which appeared to contain 75% α -copaene and 11% α -ylangene by GLC analyses. HPLC of C5 effectively separated the two closely related sesquiterpenes (Table 2) to give a +++ biologically active substance (95.6% α -copaene by capillary GLC analysis), whose mass spectral fragmentation matched that of α -copaene in the NIH/EPA Mass Spectral Library. α -Copaene samples of 94.5% and 94.8% purity (capillary GLC analyses) prepared from α -copaene-enriched samples isolated from angelica seed oil and copaiba oil, respectively, were prepared by this method. Each of these three 94.5% + pure α -copaene samples exhibited the same biological response of the same intensity for equal treatments. Due to the small observed optical rotation of α -copaene and insufficient amounts of this sesquiterpene isolated from *L. chinensis*, it was not possible to obtain a specific optical rotation.

Although there was not a sufficient quantity of the substance in C5 whose GLC retention time coincided with α -ylangene for isolation, there was an adequate source of α -ylangene in the α -copaene-enriched samples from angelica seed oil and copaiba oil. α -Ylangene, free of α -copaene, was prepared by HPLC from these samples. This α -ylangene, as well as standard α -ylangene (Table 2, 98.9% pure by capillary GLC analysis), having identical GLC and HPLC retention times, initiated the same medfly biological response of the same intensity for equal treatments; this response (++ to +++) was slightly less than that (+++) for the α -copaene for equal treatments (McInnis and Warthen, 1988).

Steam distillates of Puerto Rican and Miami *L. chinensis* also contained 1.91–2.66% (Figure 2A) and 18.39–28.47% (Figure 2B) α -copaene (retention times, 8.96 and 8.97 min, respectively), the structure of which was confirmed by GC-MS with an NIH/EPA Mass Spectral Library match for α -copaene. The overall yields of α -copaene were 0.001% and 0.0003%, respectively, agreeing with the difference in biological activity for the two samples. The Miami *L. chinensis* seemed to be devoid of a GLC peak coincident for α -ylangene, whereas the Puerto Rican *L. chinensis* steam distillate had a 0.25% peak (coincident for α -ylangene) and a 2.01% α -copaene peak, roughly 1:8.

Crude leaf stem exudates (10–50 μ l) of *F. benjamina* and *F. retusa* (McInnis and Warthen, 1988), as well as *F. benghalensis*, were each shown to attract male medflies in the laboratory bioassay. Ethyl acetate and *n*-hexane extracts of fresh leaf stem exudates of *F. benghalensis* attracted male medflies, but solvent extracts of leaf stem exudate of *F. benjamina* that were processed after shipment from Hawaii showed less activity. Therefore, it was essential to extract the leaf stem exudates immediately upon harvest.

Capillary GLC analyses of *n*-hexane, ethyl ether, ethanol, and acetone extracts of small quantities of *F. benjamina* and *F. benghalensis* leaf stem exudate did not reveal useful data due to the low concentration of terpenes.

A larger quantity of exudate was then obtained from a large specimen of *F. retusa*; the leaf stem exudate was more attractive to medflies than the trunk exudate (McInnis and Warthen, 1988). The methylene chloride extracts of the fresh exudates were biologically active to medfly and maintained this activity over time. Open-column chromatography, via Sep-Pak (to remove latexlike materials) and 20% silver nitrate–Bio-Sil A, gave a fraction that showed medfly activity analogous to B3–B4 in Scheme 1 and that contained a real peak coincident with the retention time of standard α -copaene by comparison with a blank open-column chromatographic run.

To show the presence of α -copaene in *F. retusa* (307.3 g) and *F. benjamina* (323.9 g) and by inference in their exudates, the steam distillates (48.9 g and 40.6 g of material, respectively) were analyzed by GLC. Chromatograms of the distillates (Figures 2C and 2D) indicated the presence of 21.0% α -copaene in *F. retusa* and 11.0% α -copaene in *F. benjamina* (retention times, 8.95 and 8.96 min, respectively); structure was confirmed in each case by GC-MS with an NIH/EPA Spectral Library match for α -copaene. If α -ylangene were present in these distillates, it would be present at <0.6%, based on coincident retention times with standard α -ylangene. The yield, based on dried plant specimens, of α -copaene was 1.146 mg (0.0004%) from *F. retusa* and 0.802 mg (0.0002%) from *F. benjamina*.

Capillary GLC analysis did not reveal the presence of α -copaene in extracts of a small specimen of fresh *F. benghalensis* leaves; in all probability, this was

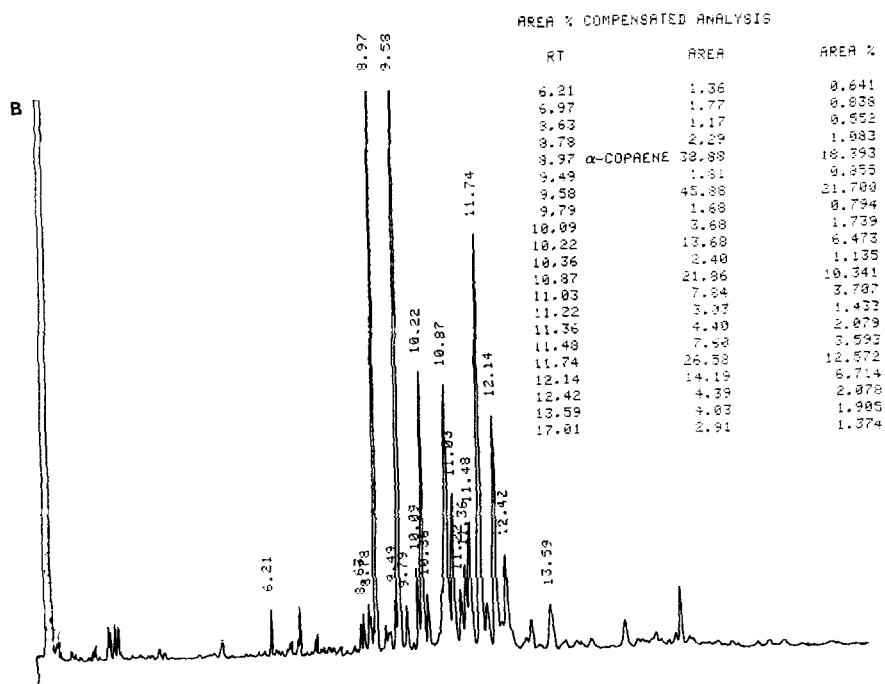
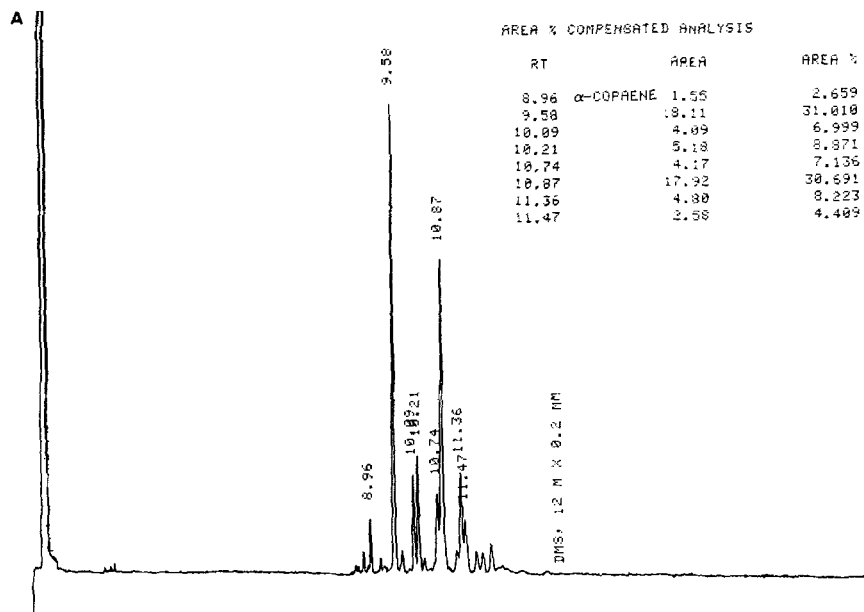


FIG. 2. Capillary GLC of the essential oils from Puerto Rican *Litchi chinensis* dried stems (A); Miami *L. chinensis* dried stems (B); *Ficus retusa* dried leaves, small stems, and twigs (C); and *F. benjamina* dried leaves, small stems, and twigs (D).

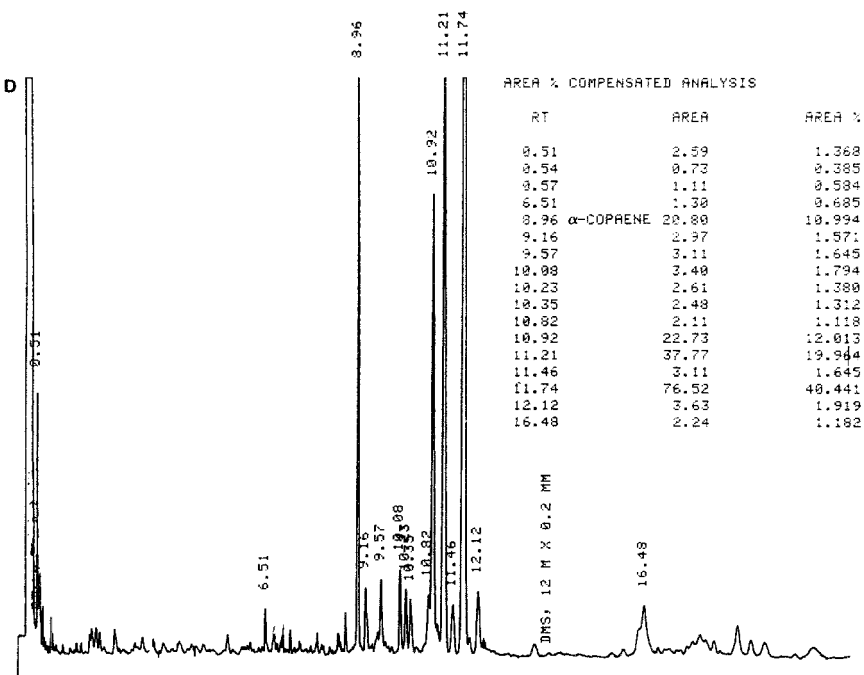
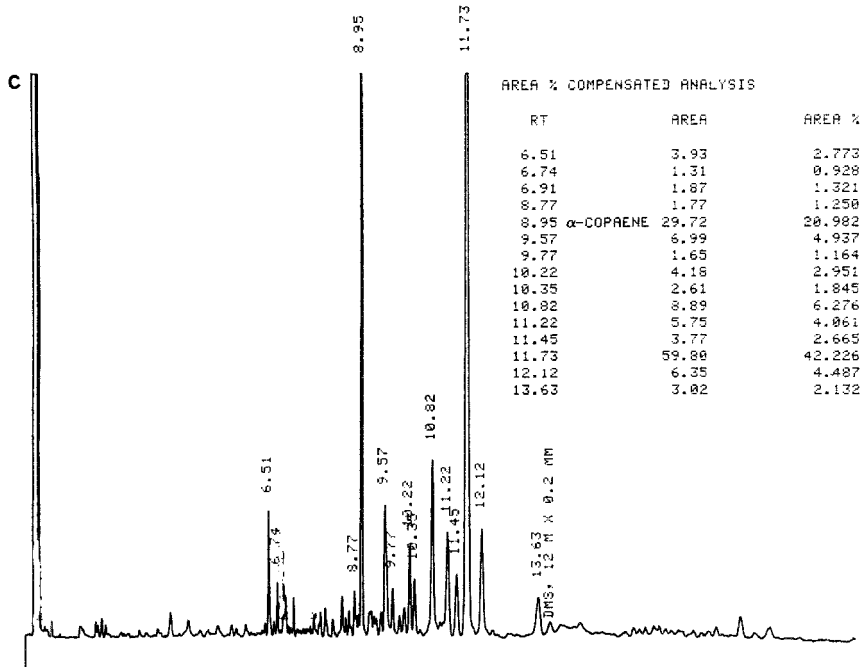


FIG. 2. Continued

due to the size of the specimen extracted. An adequate amount of this species could not be located for extraction; but, it is very likely that the attractiveness of its leaf stem exudate is also due to the presence of α -copaene.

DISCUSSION

Fornasiero et al. (1969) isolated α -copaene and α -ylangene (20:1) from angelica seed oil and demonstrated equal attractiveness to male medflies in lab bioassays (Guiotto et al., 1972); α -pinene and α -phellandrene from this oil were slightly attractive, and other sesquiterpenic fractions were more strongly attractive. The structure of α -copaene was resolved by de Mayo et al. (1965) and Kapadia et al. (1965), and the structure of α -ylangene was resolved by Motl et al. (1965) and Ohta and Hirose (1969).

α -Copaene was first isolated from African copaiba oil (Kapadia et al., 1965; Wenninger et al., 1967a,b; Lawrence, 1980); it was also isolated from *Cedrella toona* (de Mayo et al., 1965; Kapadia et al., 1965), chloranthus oil (de Mayo et al., 1965), ylang-ylang oil (Wenninger et al., 1966, 1967b), peppermint oil (Wenninger et al., 1967b), grapefruit and orange oils (Hunter and Brogden, 1964; Veldhuis and Hunter, 1968; Teranishi et al., 1987), and cubeb oil (Ohta et al., 1968; Teranishi et al., 1987). More recent reports indicate its presence in *Yucca gloriosa* (Wang and Kameoka, 1977), guava (MacLeod and de Troconis, 1982), juniper berry and oil of sage (Formacek and Kubeczka, 1982), pineapple fruits (Berger et al., 1983), leaves of *Siparuna guianensis* (Aubl.) (Antonio et al., 1984), corn leaf volatiles (Buttery and Ling, 1984), wheat (Buttery et al., 1985), cotton (Elzen et al., 1985), *Lippia nodiflora* (L.) Greene (Elakovich and Stevens, 1985), and *Heracleum dissectum* Ledeb. (Papageorgiou et al., 1985). α -Copaene usually exists as the levorotatory enantiomer; however, Jacobson et al. (1984) reported that α -copaene from angelica seed oil is dextrorotatory (Jacobson et al., 1987). Teranishi et al., (1987) also reported that the α -copaene from orange oil was dextrorotatory.

α -Ylangene has been isolated from *Schizandra chinensis* (Turcz) Baill by Motl et al. (1963) and Ohta et al. (1968), and traces have been reported in corn leaf volatiles (Buttery and Ling, 1984) and numerous other sources.

Racemic α -copaene was first synthesized via a 17-step route by Heathcock (1966) who, with Heathcock et al. (1967), reported the synthesis of racemic α -ylangene. Subsequently, Corey and Watt (1973) reported simpler syntheses of racemic α -copaene and α -ylangene.

The short-range attraction/feeding stimulation of male medflies to a host plant, *L. chinensis*, from Puerto Rico (McInnis and Warthen, 1988) was demonstrated and shown to be caused by 0.001% α -copaene in the dried twigs. Smaller amounts of a substance with a GLC retention time coincident to that of

α -ylangene, in the steam distillate (Figure 2A), also probably contributes to the behavioral response of male medflies since the α -ylangene standard (Table 2) elicits a similar response. The ratio of α -copaene to α -ylangene (retention times, 8.96 and \sim 8.83 min, respectively) in the steam distillate was 8:1. Through parallel bioassay/isolation of fractions from *Litchi* (Scheme 1), α -copaene (95.6%, α -ylangene-free) was isolated by HPLC as an active component. The behavioral response to this sample was identical to those produced by α -copaene samples (α -ylangene-free), each with a purity of 94.5% and 94.8%, prepared by HPLC of α -copaene-enriched samples from angelica seed oil and copaiba oil, respectively. No difference in behavioral response was noted to the purified α -copaene from copaiba and the purified α -copaene from angelica, each having opposing rotations, as reported by Jacobson et al. (1984, 1987); the implication is that optical rotation of these enantiomers is not relevant over short distances. α -Ylangene standard (98.9%) and α -ylangene (α -copaene-free) samples, the latter being prepared from α -copaene-enriched samples from angelica seed oil or copaiba oil, gave the same male medfly response but slightly less intense than with α -copaene. The presence of α -ylangene in *L. chinensis* from Miami was not detectable, even though there was 18.4% α -copaene (Figure 2B, retention time, 8.97 min) in the steam distillate as compared to 2.7% α -copaene (Figure 2A, retention time, 8.96 min) in the Puerto Rican sample. However, the overall yield of α -copaene was three times greater from the Puerto Rican sample than from the Miami sample. β -Caryophyllene in *L. chinensis* (B3, Scheme 1) was ruled out as a medfly attractant through bioassay even though it had been reported as an attractant (Beroza and Green, 1963a). Old samples of β -caryophyllene were probably attractive because of the presence of α -copaene as an impurity.

The short-range attraction/feeding stimulation of male medflies to the milky exudate of a nonhost plant, *F. retusa* (McInnis and Warthen, 1988), was also shown to be caused by α -copaene through a fractionation similar to Scheme 1. Fractions that would correspond to B3–4 showed a real GLC peak corresponding to α -copaene; and a steam distillate of leaves, small stems, and twigs revealed the presence of 21.0% α -copaene (Figure 2C, retention time, 8.95 min). A similar behavioral response to the milky exudate of the other nonhost plant, *F. benjamina* (McInnis and Warthen, 1988), was also probably due to α -copaene since a similar steam distillate revealed the presence of 11.0% α -copaene (Figure 2D, retention time, 8.96 min). If we had had a large enough sample of *F. benghalensis* to do a steam distillation, α -copaene would probably have been revealed as the component in the milky exudate responsible for eliciting a similar behavioral response of male medflies. If α -ylangene were present in *F. retusa* and *F. benjamina*, contributing to the medfly behavioral response, it would be present in the steam distillate (Figures 2C and 2D) at $<0.6\%$ based on coincident retention times.

The discovery of synthetics or phytochemicals as short-range attractants/feeding stimulants for the male medfly is an important area of research. Such materials may be useful in studying this short-range phenomenon and may even be used as an eradication tool when combined with a bait-toxicant system.

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SUPPRESSION OF PLANT PARASITIC NEMATODES IN THE CHINAMPA AGRICULTURAL SOILS

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Abstract—Soil from the chinampa agricultural system in the Valley of Mexico suppressed damage by plant-parasitic nematodes to tomatoes and beans in greenhouse and growth chamber trials. Sterilization of the chinampa soil resulted in a loss of the suppressive effect, thereby indicating that one or more biotic factors were responsible for the low incidence of nematode damage. Nine organisms were isolated from chinampa soil, which showed antinematodal properties in culture. Naturally occurring populations of plant-parasitic nematodes were of lower incidence in chinampa soil than in Chapingo soil.

Key Words—Nematode suppressive soil, false root knot nematode, *Nacobus aberrans*, *Meloidogyne incognita*, nematocidal microorganisms, reproduction suppression

INTRODUCTION

The chinampa agricultural system, which is based on land reclaimed from swamps and shallow lakes in the valley of Central Mexico, reached its greatest development during the reign of the Aztecs (Armillas, 1971). Records from the time of the Spanish conquest indicate that chinampas have been maintained continuously for about 2000 years (Coe, 1964); however, modern urban development has severely constricted the extent of these reclaimed farmlands. The high productivity of the chinampa system is well documented and is due to the large amounts of vegetation and sediments continuously dredged from the adja-

cent canals and added to the fields, the adequate supply of water from the canals, and the regular use of animal manures (Coe, 1964; Gliessman et al., 1981).

Casual observations indicating that root diseases were of low incidence in lands under chinampa management were confirmed through cooperative studies by Mexican and American scientists. This work demonstrated that damping-off caused by *Pythium* spp. was suppressed in chinampa soils as compared to Chapingo soils. (Lumsden et al., 1987). The Chapingo soils were used by Lumsden et al. (1987) and in the current work for comparison with the chinampa soils since disease suppression has been reported from chinampa but not Chapingo soils, yet both occur in the same geographical area. The current paper details experiments on the incidence of a nematode-induced disease as affected by the chinampa system, using for comparison soils from experimental fields located at the Postgraduate College, Chapingo, Mexico, and studies on the possible mechanisms underlying these effects.

METHODS AND MATERIALS

Greenhouse Trials. The influences of the chinampa and Chapingo soils on plant growth and root galling caused by the false root knot nematode *Nacobbus aberrans* and the root knot nematode *Meloidogyne incognita* were first evaluated in two greenhouse trials (see Tables 1 and 2). Host plants were tomato (*Lycopersicon esculentum* var. Ace) and bean (*Phaseolus vulgaris* var. Flor de Mayo) for the false root knot nematode experiment and tomato for the root knot

TABLE 1. GREENHOUSE TRIAL ON EFFECTS OF FALSE ROOT KNOT NEMATODE (*Nacobbus aberrans*) ON TOMATO AND BEAN PLANTS IN CHINAMPA AND CHAPINGO SOILS

Soil type and treatment ^a	Tomato ^b			Bean ^b		
	Top wt (g)	Root wt (g)	Gall index	Top wt (g)	Root wt (g)	Gall index
Chapingo						
Sterilized	30 a	11 c	5 d	53 f	9 h	4 i
Nonsterilized	37 a	10 c	4 d	49 f	8 h	4 i
Chinampa						
Sterilized	40 a	8 c	4 d	57 f	9 h	3 j
Nonsterilized	63 b	9 c	2 e	65 g	7 h	2 k

^a Each treatment was replicated eight times. Experiment duration was 63 days.

^b Values for each host followed by different letters are significantly different ($P < 0.05$) from the other, according to Duncan's multiple-range test.

TABLE 2. GREENHOUSE TRIAL ON EFFECTS OF ROOT KNOT NEMATODE (*Meloidogyne incognita*) ON TOMATO PLANTS IN CHINAMPA AND CHAPINGO SOILS^a

Soil type and treatment	Dry weight (g)		Gall index
	Top	Root	
Chapingo			
Sterilized	2.8 a	0.42 b	4.0 d
Nonsterilized	2.7 a	0.37 b	4.0 d
Chinampa			
Sterilized	3.0 a	0.45 b	3.2 d
Nonsterilized	3.4 a	0.53 c	1.5 e

^aEach treatment was replicated eight times. Experiment duration was 63 days. Values for each treatment followed by different letters differ significantly ($P < 0.05$) from each other.

nematode experiment. Soils used were from the chinampa system and from Chapingo. Soils were sterilized by steam or were unsterilized. There were eight replicates of each treatment (the total number of pots in each experiment was 64). The ambient temperature was 20–27°C. These trials and others involving the processing of soil were performed in Chapingo.

The soils were the same as used by Lumsden et al. (1987). The physical and chemical characteristics of the chinampa and Chapingo soils were examined in detail by these workers, and their data are cited in the discussion of the current results.

Each pot contained 400 cc of soil and one plant. Three weeks after planting, a gram of nematode-infested roots was added to each pot. In the *N. aberrans* experiment, the plants were harvested after nine weeks and the wet weight of the plant roots and tops recorded. Growth of plant roots and tops was evaluated as dry weight in the *M. incognita* experiment. The number of galls on the roots were counted and were rated by a gall index as follows: no galls = 0; 1–2 galls = 1; 3–10 galls = 2; 11–30 galls = 3; 31–100 galls = 4; more than 100 galls = 5. Statistical analyses of results were by Duncan's multiple-range test.

Growth Chamber Trial. The influence of the chinampa and Chapingo soils on nematode attack was also evaluated in the growth chamber (see Table 3). Parameters were the same as for the greenhouse trial, with the following exceptions. Another category of chinampa soil was tested, specifically chinampa soil with fungicide and insecticide residues resulting from chemical pesticide treatment. The plant host tested was Ace tomato. Each treatment was replicated 10 times (total number of pots = 50). Growth chamber conditions were $23 \pm 1^\circ\text{C}$, 14 hr light and 10 hr dark. Duration of the experiment was 75 days.

TABLE 3. GROWTH CHAMBER TRIAL ON EFFECTS OF FALSE ROOT KNOT NEMATODE (*Nacobbus aberrans*) ON TOMATO PLANTS IN CHINAMPA AND CHAPINGO SOILS^a

Soil type and treatment	Plant weight (g)		Gall index
	Top	Root	
Chapingo			
Sterilized	45 a	8 c	4 d
Nonsterilized	50 a	6 c	4 d
Chinampa			
Nonsterilized, fungicides and insecticides used	47 a	5 c	2 e
Sterilized	39 a	8 c	3 d
Nonsterilized	60 b	6 c	1 f

^aEach treatment replicated 10 times. Experiment duration was 75 days. Values for each treatment followed by different letters differ significantly ($P < 0.05$) from each other.

Isolation of Soil Microorganisms. Chinampa soils were sampled at two separate dates 10 months apart. The soil was evaluated for bacteria, actinomycetes, and fungi following protocols detailed below. The procedure yielded a total of 466 pure cultures, many of which were undoubtedly the same species. No attempts at identification were made prior to determination of nematicidal or other antinematodal activity. Procedures were as follows: One gram of chinampa soil was shaken vigorously in 100 ml sterile 0.9% NaCl and further diluted to give concentrations of 1×10^{-6} , 1×10^{-7} , and 1×10^{-9} . An 0.2-ml aliquot of these three dilutions was then applied to four Petri plates each of (1) potato dextrose agar, (2) nutrient broth agar, (3) glucose-starch-asparagine agar, (4) glycerol-arginine agar, (5) glycerol-casein agar, (6) glycerol-starch-peptone agar, (7) glycerol synthetic agar, and (8) yeast starch agar (Anonymous, 1975). Isolates were subcultured at 3 and 15 days after plating to obtain pure cultures. Each isolate was spread in two thin lines across a 9-cm Petri plate containing the medium on which the original culture was isolated, so as to divide the plates into four equal quadrants. After three days of growth at 25°C, 30 μ l of broth containing *Escherichia coli* was placed in the center of three quadrants, and 20 μ l saline containing 30–50 of the nematode *Caenorhabditis elegans*, which had been axenically grown on heme soybean extract-yeast extract medium (McClure and Zuckerman, 1982), was added to the center of the fourth quadrant. Exudates of *E. coli* are strongly attractive to *C. elegans* (Jansson et al., 1986); thus the assay served to induce the nematodes to cross the barrier formed by the test isolate. The numbers of nematodes that had reached the *E. coli* were counted

at 3 and 24 hr, and a count was made of the numbers that stayed in the strip of the test isolate. Nematicidal activity was indicated when the nematodes died, and repellency when the nematodes lived but did not cross (or enter into) the barrier formed by the test isolate. Additional information on attraction and repellency was obtained using 3-day-old liquid cultures of bacteria in nutrient broth or 3- to 5-day-old liquid cultures of fungi in potato dextrose broth. Eight-microliter aliquots of culture extract were added to water agar plates, the plates equilibrated overnight and numbers of *C. elegans* migrating under the agar disks counted 60 min after addition of the worms as described by Jansson et al. (1984). In addition, where nematicidal activity occurred, tests included second-stage larvae of *M. incognita* collected by the method of Hussey and Barker (1973).

All isolates were then incubated for one week at 22°C and reexamined to determine if the nematodes were breeding normally or if reproduction was delayed or inhibited. A culture of *Streptomyces avermitilis* (ATCC 31267), which showed nematicidal activity, and a culture of *Aspergillus phoenicis* (ATCC 14332), which was both toxic and repellent, to nematodes, served as standards.

Incidence of Soil Nematodes. Samples of chinampa and Chapingo soils were examined to yield an estimate of the numbers of plant parasitic, bacteriophagous, and predaceous nematodes present. Each sample consisted of 100 cm³ of soil processed by sieving and use of the Baermann funnel. After 24 hr, an aliquot of water was drawn from the Baermann funnel and the nematodes counted and identified.

RESULTS

Greenhouse Trials. The greenhouse trial with the false root knot nematode *N. aberrans* showed significantly ($P < 0.05$) less root galling of both tomato and bean plants in nonsterilized chinampa soil as compared to nonsterilized and sterilized Chapingo soil (Table 1). The sterilized chinampa soil and the nonsterilized Chapingo soil showed significant differences in gall index, but the level of galling in the nonsterilized chinampa soil was much lower than that in the sterilized chinampa soil. Growth of the bean and tomato plants, as measured by the wet weight of the aboveground portions of the plants, was significantly enhanced, but there was no consistent difference in root growth. Sterilized chinampa soil had greatly reduced nematode suppressive activity, indicating that the reduction of nematode attack is related to biotic factors.

The root knot nematode *M. incognita* caused significantly less root galling ($P < 0.05$) on tomato in chinampa nonsterilized soil than in the other treatments (Table 2). These results support those of other experiments reported here that

biotic factors were responsible for the reduction in nematode damage. In this experiment root growth was enhanced in the nonsterile chinampa soil, but top growth was not significantly affected.

Growth Chamber Trial. The results of the growth chamber experiment supported those obtained in the greenhouse (Table 3). Root galling was significantly less ($P < 0.05$) and the vegetative growth of plants significantly greater ($P < 0.05$) in the nonsterilized chinampa soil than in the sterilized chinampa soil. The Chapingo soil, both sterilized and nonsterilized, showed no nematode suppression. Chinampa soil that was not sterilized but that was treated with agricultural pesticides was less suppressive to nematode attack and growth of plants was reduced. These results support a role for a biotic factor in enhanced plant growth and reduction in nematode attack associated with the chinampa agricultural system.

Antinematodal Organisms from Chinampa Soil. Of the 466 organisms from the chinampa soil, nine isolates were active against *C. elegans* (Table 4). The two fungi isolated on potato-dextrose agar were identified as belonging to the *Aspergillus niger* and *A. ochraceus* groups. Culture filtrates of the *A. niger* isolate that passed through a dialysis membrane with a 6000 mol wt cutoff, killed *C. elegans* within 10 min. These filtrates were toxic to newly hatched second-stage larvae of *M. incognita* within 60 min. Eight-microliter aliquots of

TABLE 4. ANTINEMATODAL ACTIVITY AGAINST *C. elegans* OF MICROORGANISMS ISOLATED FROM CHINAMPA SOIL

Medium ^a	Bacteria		Actinomycetes		Fungi	
	No. isolated	No. active	No. isolated	No. active	No. isolated	No. active
1	45	1 ^b	1	0	4	2 ^c
2	57	5 ^b	1	0	4	0
3	65	0	6	0	8	0
4	58	0	3	0	1	0
5	33	0	1	0	3	0
6	62	0	2	0	0	0
7	38	0	1	0	4	1 ^c
8	62	0	5	0	2	0

^aMedia were: (1) potato-dextrose agar; (2) nutrient broth agar; (3) glucose-starch-asparagine agar; (4) glycerol-arginine agar; (5) glycerol-casein agar; (6) glycerol-starch-peptone agar; (7) glycerol synthetic agar; (8) yeast-starch agar.

^bInhibited reproduction.

^cNematicidal.

filtrates from this culture, allowed to diffuse in water agar plates overnight, were highly repellent to *C. elegans*. The *A. ochraceus* group isolate was slower acting, but still highly toxic to *C. elegans*. A third fungus, isolated on medium 7, was also nematocidal to *C. elegans*. This organism has not as yet been identified, but the red pigment it produces suggests it may be a *Fusarium* sp. Six bacteria isolated on medium 1 and medium 2 inhibited reproduction of *C. elegans*. The criteria for inhibition of reproduction was that seven days after 30 nematodes were placed on a plate, either no reproduction had occurred or there were less than 50 nematodes/plate. By comparison, plates showing no inhibition of reproduction contained more than 1×10^5 nematodes.

Incidence of Soil Nematodes. The results of soil sampling of the chinampa and Chapingo soils demonstrated that numbers of both economically important plant parasitic nematodes and other plant nematodes were lower in chinampa soil than in Chapingo soil (Table 5). The large numbers of *Acrobeles* sp. and *Rhabditis* sp. agree with other observations (Sayre, 1971) that soils high in organic matter support high populations of these bacteriophagous nematode species.

TABLE 5. POPULATIONS OF SOIL NEMATODES IN CHINAMPA AND CHAPINGO SOILS^a

Nematode group	Soil type			
	Chinampa		Chapingo	
	Genus	No./100 cm ³ soil	Genus or species ^b	No./100 cm ³ soil
Economically most important plant parasitic nematodes	<i>Pratylenchus</i>	3	<i>Pratylenchus</i>	17
			<i>Nacobbus</i> <i>aberrans</i>	2
Other plant parasitic nematodes	<i>Criconemella</i>	15	<i>Hoplolaimus</i>	40
	<i>Nothocriconema</i>	11	<i>Tylenchorhynchus</i>	38
	<i>Tylenchorhynchus</i>	8	<i>Tylenchus</i>	40
	<i>Ditylenchus</i>	4		
	<i>Aphelenchus</i>	5		
Predaceous nematodes	<i>Tylenchus</i>	6		
	<i>Dorylaimus</i>	8	<i>Dorylaimus</i>	4
Bacteriophagous nematodes	<i>Rhabditis</i>	30	<i>Rhabditis</i>	15
	<i>Acrobeles</i>	40		

^aData derived from averages of three 100-cm³ samples.

^bNematodes were identified only to genus, except for *N. aberrans*.

DISCUSSION

Observations by Lumsden et al. (1987) on disease suppression in chinampa soil will be discussed in the context of the current study. An important observation was the increased incidence of disease caused when inoculum of *Pythium aphanidermatum* was added to both Chapingo and chinampa soils after the soil was subjected to gamma ray sterilization (Lumsden et al., 1987). This finding implicates a biotic factor in the disease suppression observed in chinampa soils. The present finding of enhanced susceptibility to nematodes in steam sterilized chinampa soil also suggests a crucial role for biotic factors in suppression of nematode damage in chinampa soil.

Lumsden et al. (1987) give a comprehensive analysis of the chemical and physical characters of the Chapingo and chinampa soils. They found that the chinampa soil was a silty, clay loam, pH 5.8, and was significantly higher in organic matter and P, K, Ca, Mg, NO₃, and NH₄ content than the Chapingo soil, as well as soluble salts. Chapingo soil, in addition to being lower in organic matter, was sandy and had a pH of 6.8. It is recognized that organic amendments to soil result in a shift in microbial populations, due to factors probably including more acid pH and the production of toxic materials. Sayre (1971) summarized the numerous observations that confirm significant reductions of phytonematodes in soils that conform to the chinampa profile. The high organic content of chinampa soil is probably partly responsible for the paucity of phytonematodes and the suppression of plant damage, but the importance of soil organisms in the reduction of injury by plant nematodes is also emphasized by the current experiments.

Of the nine organisms that showed antinematodal activity, the *A. niger* group isolate alone has been tested against a plant parasitic nematode. This fungus produced exudates that were highly nematocidal to both *C. elegans* and *M. incognita* at high concentrations and repellent to *C. elegans* at lower concentrations. Greenhouse tests to evaluate the efficacy of this organism against root knot nematodes are planned. Further evaluation will also proceed on the organisms that showed lower nematocidal activity or effects that retarded nematode development. Our interpretation of the current results is that the sum of effects of these nine organisms is probably large enough to account for the suppression of plant nematode damage observed in the chinampa soil.

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ALLELOPATHIC PROPERTIES OF *Polygonella myriophylla* Field Evidence and Bioassays

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Abstract—*Polygonella myriophylla* is a perennial shrub endemic to the Florida scrub. Striking bare zones surround mature *Polygonella* stands. Quantitative measurements of root distribution show that few *Polygonella* roots extend into the bare zones, supporting the hypothesis that the bare zones result from chemical interference by *Polygonella* with the growth of other species. Bioassays of soils collected biweekly for one year from beneath *Polygonella*, the bare zone, and adjacent grassed areas confirm that the germination and growth of grasses is reduced in *Polygonella* soil and soil from the surrounding bare zone. Compared to adjacent grassed area soil, the average germination of bahiagrass (*Paspalum notatum*) was 71% in *Polygonella* soil and 81% in bare zone soil, and average shoot dry weight was 48% in *Polygonella* soil and 81% in bare zone soil. Seasonal variation in the inhibition of grass germination and growth was not pronounced.

Key Words—*Polygonella myriophylla*, *Paspalum notatum*, allelopathy, interference, sand pine scrub.

INTRODUCTION

The sand pine scrub community occurs on well-drained sandy soils along Florida's central ridge and coastal dunes. At many sites, sand pine, *Pinus clausa* (Chapm. ex Engelm.) Vasey ex Sarg., and oaks, including *Quercus chapmanii* Sarg., *Q. myrtifolia* Willd., and *Q. geminata* Small, form a dense canopy with little or no herbaceous understory. More open sites are dominated by widely

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spaced evergreen shrubs, such as *Ceratiola ericoides* Michx., *Conradina canescens* (Torr. & Gray) A. Gray, and *Polygonella myriophylla* (Small) Horton, with scattered young scrub oaks, sand pine, and scrub palmetto, *Serenoa repens* (Bartr.) Small. Few plants grow in the large open areas between shrubs. These include *Paronychia chartacea* Fern., a small plant less than 10 cm in height, and *Selaginella arenicola* Underw., a club moss. In many areas the soil is covered by a mat of lichens 2.5–15 cm thick.

Where open scrubs abut roads or abandoned fields, sharp boundaries between the scrub and neighboring vegetation often are evident (Figure 1). Grasses such as bahiagrass, *Paspalum notatum* Fluegge, and natalgrass, *Rhynchelytrum repens* (Willd.) C.E. Hubb., and the composites camphorweed, *Heterotheca subaxillaris* (Lam.) Britt & Rusby, and dog fennel, *Eupatorium capillifolium* (Lam.) Small, are common along roadsides but generally do not occur in the scrub.

In past years, similar sharp ecotones marked by a persistent bare zone 1–2 m wide were reported between the sand pine scrub and the sandhill, another community that occurs on well-drained sandy soils (Nash, 1895; Harper, 1921; Mulvania, 1931; Webber, 1935). Sandhills are dominated by longleaf pine,



FIG. 1. Patches of *Polygonella myriophylla* (left) dominate portions of a scrub near Sun Ray, Florida, which borders an abandoned citrus field that has been invaded by bahiagrass, *Paspalum notatum*, and other ruderal species. The bare zone is approximately 1 m wide.

Pinus palustris Mill., slash pine, *P. elliotii* var. *densa* Little & Dorman, and oaks, *Q. laevis* Walt. and *Q. geminata*. In contrast to the scrub, wiregrass, *Aristida stricta* Michx., other grasses and many herbs form a fairly dense herbaceous ground cover in the sandhill.

The possible role of allelopathy in the scrub is beginning to be explored (Richardson, 1985; Tannrisever et al., 1987; Richardson and Williamson, 1988). Richardson (1985) demonstrated that leaf washes of several plants, including *Polygonella myriophylla*, inhibited germination and growth of lettuce, *Lactuca sativa* L., and the native sandhill grasses *Andropogon gyrans* Ashe, *Schizachyrium scoparium* (Michx.) Nash and *Leptochloa dubia* (H.B.K.) Nees.

The experiments reported here were performed to test the hypothesis that the dominance of *Polygonella myriophylla* in certain areas of the Florida scrub is due in part to allelopathic interference. A study of the distribution of root mass around a mature *Polygonella* stand was carried out to determine whether the striking bare zone observed extends beyond the *Polygonella* root zone. Bioassays were conducted with soil collected biweekly for one year from beneath and around *Polygonella* shrubs to determine whether there is seasonal variation in the phytotoxicity of *Polygonella* soil.

METHODS AND MATERIALS

Root Mass Distribution Study

Site Description. South of Lake Wales, Florida, in a vacant field across from Warner Southern College on the west side of U.S. Route 27 (Range 27E, Township 30S, NW Quarter of Section 35), an area of open scrub was found that had been disturbed several years ago. Areas of scrub are now intermixed with patches of ruderal species, including bahiagrass (*Paspalum notatum*), natalgrass (*Rhynchelytrum repens*), and dog fennel (*Eupatorium capillifolium*). One large patch of *Polygonella myriophylla* is more than 4 × 9 m in extent. It borders an area of bahiagrass and is surrounded by a bare zone 0.6–1.0 m wide. Dead or dormant rhizomes of bahiagrass are found within the patch and in the bare zone.

Sampling and Root Mass Measurements. To measure the distribution of root mass across the bare zone, soil samples were taken in April 1985 from four areas: within the large *Polygonella* patch described above, at the edge of the patch, in the middle of the bare zone, and 1 m into the adjacent stand of bahiagrass. In each area, five soil cores (10 cm diameter × 20 cm depth) were obtained at random points. Roots were separated from the soil by sieving through a 2-mm screen, sorted by species, and dried for 48 hr at 80°C. Roots of *Polygonella myriophylla* and *Paspalum notatum* were easily distinguished from one another by their different color and morphology.

Study of Seasonal Variation of Soil Phytotoxicity

Site Description. The study area is located at the north end of the Lake Arbuckle scrub, approximately five miles southeast of Frostproof, Florida, off Rucks Dairy Road (Range 29E, Township 32S, south half of Section 8). The site is owned by the state of Florida and administered by the Division of Forestry. At this site, *Polygonella*-dominated scrub borders both sides of an old road cut for more than 250 yards. A dense bahiagrass cover 1–2 m on either side of the road does not extend into the scrub, producing sharp boundaries similar to that seen in Figure 1. Lichen cover (*Cladonia* and *Cladina* spp.) at the site is sparse.

Sampling Protocol. Samples of *Polygonella*, bare zone, and grassed area soils were collected biweekly from November 1984 to October 1985. The study area was initially mapped to determine the number of *Polygonella* patches suitable for sampling. There were over 200 patches greater than 1 m in diameter chosen, more than 100 on each side of the road. The patches were numbered, and at each sampling date, four patches, two on either side of the road, were randomly selected.

Collection of Soils. Soil samples were collected from directly beneath *Polygonella* patches, in the bare zones 0.6 m distant from the patches, and in neighboring grassed areas. Three 5-cm-diameter cores of the upper 5 cm of each soil type were collected at each of the four sampling sites. Soils collected at sampling sites 1 and 3 and sites 2 and 4 were combined to give two replicates of each soil type per sampling date.

A thin layer of litter (<0.5 cm) covers the soil beneath *Polygonella* stands. The texture of this litter layer was fine and difficult to separate from the underlying soil. Therefore, litter was not removed prior to sampling.

Storage and Preparation of Samples. All soil samples were stored frozen in polyethylene bags for a maximum of eight months (–20°C). Results of preliminary bioassays did not indicate any differences in the toxicity of freshly collected and frozen soils. Prior to the bioassays, soil samples were air-dried, passed through a 2-mm sieve to remove roots and other large debris, and mixed thoroughly.

Rainfall Measurements. Daily rainfall measurements were recorded within 1 km of the study site for the duration of the study.

Bioassay Methods. Bioassays were conducted in a large, walk-in growth chamber. Day length was 12 hr and light intensities at pot level were approximately 350 $\mu\text{mol}/\text{m}^2/\text{sec}$. Maximum daytime temperatures were 25–28°C, and night temperatures were 20–22°C. Humidity was not controlled. Fifty seeds of bahiagrass, *Paspalum notatum*, or the native sandhill grass little bluestem, *Schizachyrium scoparium*, were sown in 5 × 5-cm pots each containing 200 g soil. Each soil sample (two each of *Polygonella*, bare zone, and grassed area soil

per sampling date) provided soil for four pots, two replicates each of bahiagrass and little bluestem. Because of the large volume of soil involved, soil collected in October 1984 through mid-April 1985 and late April through October 1985 was assayed in separate experiments.

The arrangement of pots was randomized weekly. Pots were watered twice weekly to 12.5% moisture content, slightly below field capacity, to preclude leaching of phytotoxins from the soil. Moisture content of the pots was determined by weighing approximately 20 randomly selected pots before each watering.

Germination percentage was determined at three weeks. Plants were then thinned to two of the largest seedlings per pot by carefully pulling out the extra seedlings. This density was maintained by weekly thinnings until the plants were harvested at 6 weeks. At harvest, the number of leaves and length of the longest leaf of each seedling were recorded. Shoots were clipped at the point of the first root, and total shoot fresh weight per pot was determined. Shoot dry weight was measured after drying 48 hr at 80°C.

Soil and Data Analyses

Soil Mineral Analyses. Samples were sent to the Extension Soil Testing Laboratory of the University of Florida (Gainesville, Florida) for determination of soil pH, P, K, Ca, Mg, and organic matter content. Analyses for total, ammonium, and nitrate N were performed by the Soils Laboratory of Kansas State University (Manhattan, Kansas). All samples were air-dried before analysis. Soil texture determinations were made by water sedimentation rate using a soil texture kit from Forestry Suppliers, Inc. (Jackson, Mississippi).

Data Analysis. Data were subjected to standard analysis of variance procedures. In the seasonal study, the effects of soil type, sampling date, soil type \times sampling date, and sample (soil type) were tested for by this analysis. A significance level of 0.05 was used in the analysis and multiple comparison procedures.

RESULTS AND DISCUSSION

Root Mass Distribution Study

While some *Polygonella* roots extend across the bare zone, most of the root system and almost all of the fine roots are concentrated beneath the plant (Table 1). Compared to the root mass within the *Polygonella* patch, the root mass of *Polygonella* is 37% at the edge of the patch and only 3% in the bare zone. The root mass of bahiagrass is also greatly reduced in the bare zone. This strongly suggests a noncompetitive interaction between the two species

TABLE 1. ROOT BIOMASS OF *Polygonella myriophylla* AND *Paspalum notatum* WITHIN AND AROUND MATURE *Polygonella* STAND

Area sampled	Average root biomass (g) ^a			
	<i>Polygonella</i>	<i>Paspalum</i>	Other	Total
Within <i>Polygonella</i> patch	3.84 a ^b	1.25 b (100% dead)	0.00	5.09 b
Edge of <i>Polygonella</i> patch	1.43 ab	1.80 b (100% dead)	0.12	3.35 b
Middle of bare zone surrounding patch	0.13 b	4.36 b (50% dead)	0.16	4.63 b
Neighboring <i>Paspalum</i>	0.09 b	10.15 a	0.00	10.24 a

^aEach value is the average dry weight of roots in five randomly taken soil cores (10 cm diameter × 20 cm depth) from each area.

^bMeans in a column followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test. For *Polygonella* roots, $F = 9.69$, $Pr > F = 0.0007$, and for *Paspalum* roots, $F = 10.04$, $Pr > F = 0.0006$.

(see Dekker et al., 1983) and supports the hypothesis that toxins from *Polygonella* have played an important role in the displacement of bahiagrass at this site.

Soil properties across the *Polygonella*-bahiagrass ecotone are fairly uniform (Table 2). Given the evidence that *Polygonella* has displaced the bahiagrass stand over a period of years, the lower nitrogen levels beneath *Polygonella* appear to be a result rather than a cause of the observed vegetation patterning. This probably reflects differences in the nitrogen economies of the two species. Alternatively, this could be due to the influence of *Polygonella* allelochemicals on soil nitrification (Rice and Pancholy, 1974; Lodhi, 1978; Lodhi and Killingbeck, 1980) and nitrogen fixation (Rice et al., 1981). The possible influence of *Polygonella* allelochemicals on the soil nitrogen cycle needs to be investigated.

Animal activity, which has been demonstrated to contribute to bare zones around shrubs in the California chaparral (Bartholomew, 1970), is considered unlikely to cause the development of bare zones around *Polygonella* for several reasons: (1) Observations of some small mammal feces (possibly rabbit or armadillo) in grassed areas, as well as occasional diggings apparently to get at *Paspalum* roots, attest to the potential importance of animal herbivores in reducing growth of grasses. However, such activity has not been observed in the bare zones around scrub perennials. (2) Young patches of *Polygonella* at the same site, over 1 m in diameter and large enough to provide shelter for small mammals, have vigorous stands of bahiagrass growing within and around the patch.

TABLE 2. TEXTURE AND NUTRIENT CONTENT OF SOIL FROM BENEATH *Polygonella myriophylla*, BARE ZONE, AND ADJACENT GRASSED AREA

Soil properties	Lake Wales site ^a			Lake Arbuckle scrub ^a		
	<i>Polygonella</i>	Bare zone	Grassed area	<i>Polygonella</i>	Bare zone	Grassed area
Texture						
Sand (%)	93.2 NS	93.2 NS	90.6 NS	93.2 a	93.2 a	90.6 b
Silt (%)	3.2 NS	2.8 NS	2.2 NS	3.4 b	3.4 b	4.9 a
Clay (%)	3.5 ab	1.7 b	5.6 a	3.4 NS	3.4 NS	4.5 NS
pH	5.1 NS	5.1 NS	5.2 NS	5.0 a	4.8 b	5.0 a
P (ppm)	4.0 NS	4.0 NS	4.0 NS	3.3 a	1.7 b	4.0 a
K (ppm)	5.3 NS	5.3 NS	5.3 NS	6.0 ab	4.3 b	7.7 a
Mg (ppm)	12.0 NS	8.0 NS	11.0 NS	19.0 a	6.0 b	19.0 a
Ca (ppm)	53 NS	49 NS	51 NS	116 a	48 b	59 b
Organic matter (%)	0.6 NS	0.5 NS	0.5 NS	1.0 a	0.6 b	1.1 a
Total N (ppm)	176 NS	n.a.	328 NS	181 ab	144 b	306 a
NH ₄ ⁺ -N (ppm)	1.0 b	n.a.	1.6 a	2.0 b	2.7 ab	4.9 a
NO ₃ ⁻ -N (ppm)	0 NS	n.a.	0.4 NS	0.2 NS	0.5 NS	0.2 NS

^aData for the Lake Wales site are based on the average of three samples. For the Lake Arbuckle site, $N = 12$. For the soil nitrogen analyses, $N = 4$ for the Lake Wales site, and $N = 3$ for the Lake Arbuckle site. For each site, means within a row followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple-range test. NS = not significant, n.a. = data not available.

(3) Late in the rainy season, some stunted *Paspalum* shoots appear in the bare zone from the apparently dormant rhizomes. These observations are consistent with the hypothesis that reduction of bahiagrass growth around *Polygonella* patches is due to the chronic toxic effects of *Polygonella* allelochemicals or their breakdown products. A gradual buildup of soil concentrations of allelochemicals, while a possibility, seems unlikely on these well-drained sandy soils.

Study of Seasonal Variation of Soil Phytotoxicity

Germination and Growth Bioassays. Analysis of variance showed a significant effect of soil type (i.e., *Polygonella*, bare zone, and grassed area soils) on germination and growth of grasses for all but little bluestem germination ("soil" term in Table 3).

Germination and growth of bahiagrass were reduced significantly in *Polygonella* soil compared to adjacent grassed areas (Figures 2 and 3). Reductions of germination and growth were intermediate in the bare zone soil. Average germination of bahiagrass for the year (expressed as percent of grassed area soil) was 71% in *Polygonella* soil and 81% in the bare zone soil (Figure 2).

TABLE 3. SUMMARY OF ANALYSES OF VARIANCE OF GERMINATION AND DRY WEIGHT DATA FROM SEASONAL STUDY BIOASSAYS

Species and parameter	Sampling period	Source	<i>F</i>	<i>Pr</i> > <i>F</i>
Bahiagrass				
Germination	November-April	Model	2.54	0.0001
		Soil	24.32	0.0001
		Date	1.63	0.1010
		Soil × date	1.08	0.3823
		Sample (soil) ^a	2.07	0.1083
Germination	April-October	Model	2.54	0.0001
		Soil	25.49	0.0001
		Date	1.24	0.2620
		Soil × date	1.31	0.1752
		Sample (soil)	2.32	0.0786
Dry Weight	November-April	Model	4.23	0.0001
		Soil	23.00	0.0001
		Date	4.61	0.0001
		Soil × date	2.68	0.0004
		Sample (soil)	1.71	0.1689
Dry Weight	April-October	Model	7.48	0.0001
		Soil	86.58	0.0001
		Date	5.64	0.0001
		Soil × date	2.59	0.0004
		Sample (soil)	1.14	0.3355
Bluestem				
Germination	November-April	Model	3.11	0.0001
		Soil	0.49	0.6131
		Date	7.44	0.0001
		Soil × date	1.58	0.0651
		Sample (soil)	0.21	0.8902
Germination	April-October	Model	1.01	0.4684
		Soil	1.50	0.2281
		Date	0.92	0.5306
		Soil × date	1.12	0.3367
		Sample (soil)	0.19	0.9031
Dry weight	November-April	Model	2.15	0.0012
		Soil	6.86	0.0016
		Date	2.10	0.0265
		Soil × date	1.51	0.0848
		Sample (soil)	3.89	0.0112
Dry weight	April-October	Model	1.86	0.0056
		Soil	17.45	0.0001
		Date	1.44	0.1593
		Soil × date	0.88	0.6278
		Sample (soil)	0.94	0.4229

^aThe sample (soil) term represents subsample variation.

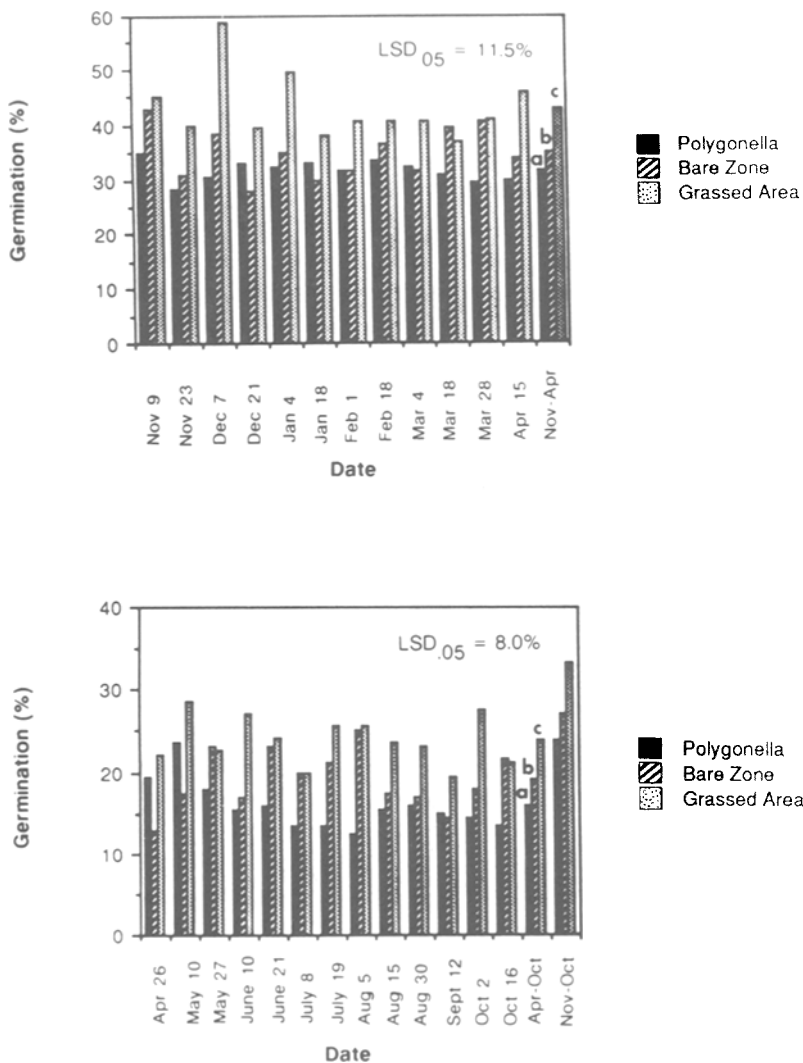


FIG. 2. Germination of bahiagrass three weeks after planting in soil collected beneath and around *Polygonella myriophylla* and from adjacent grassed areas in November 1984–October 1985. For the six-month means, significant differences are indicated by letters.

The lower overall germination rates for the April–October samples were due to slightly lower (2–3°C) temperatures in the growth chamber during the period of the experiment. Average shoot dry weight of bahiagrass was more severely affected. For the year, growth (expressed as percent of grassed area soil) was 48% in *Polygonella* soil and 81% in the bare zone soil (Figure 3).

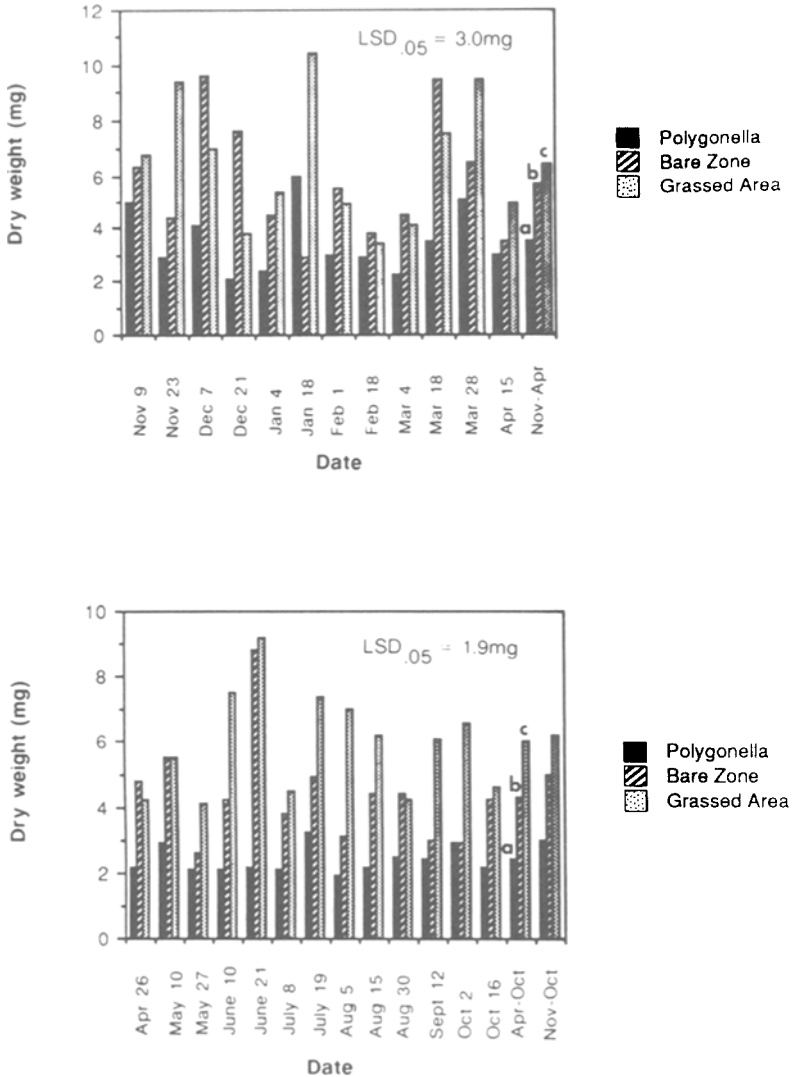


FIG. 3. Shoot dry weight of bahiagrass grown six weeks in soil collected beneath and around *Polygonella myriophylla* and from adjacent grassed areas in November 1984–October 1985. For the six-month means, significant differences are indicated by letters.

Little bluestem germination was unaffected by *Polygonella* soil (data not shown), and growth reductions were less than for bahiagrass (Figure 4). Average germination of little bluestem for the year (expressed as percent of grassed area soil) was 92% in *Polygonella* soil and 95% in bare zone soil. Average

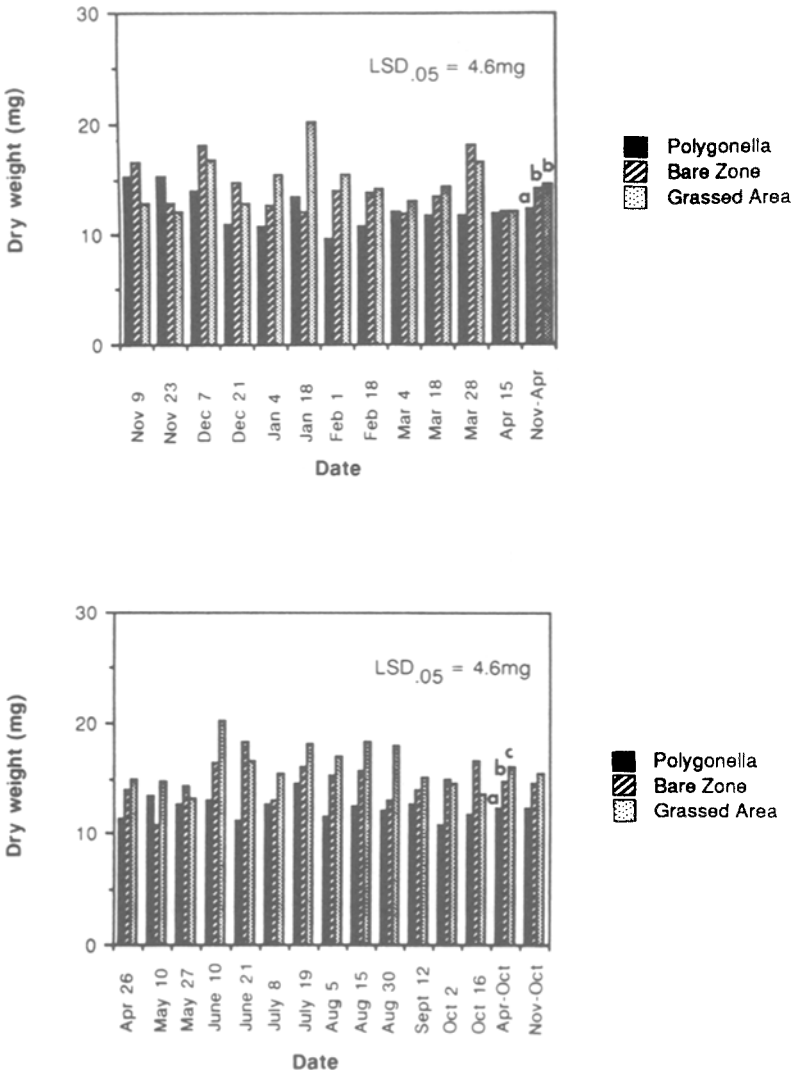


FIG. 4. Shoot dry weight of little bluestem grown six weeks in soil collected beneath and around *Polygonella myriophylla* and from adjacent grassed areas in November 1984–October 1985. For the six-month means, significant differences are indicated by letters.

shoot dry weight of little bluestem for the year (expressed as percent of grassed area soil) was 80% in *Polygonella* soil and 94% in bare zone soil (Figure 4).

It was expected that the toxicity of *Polygonella* soil would vary during the year because of the influence of environmental factors such as rainfall and tem-

perature on the processes of phytotoxin production, release, accumulation, and breakdown. Richardson and Williamson (1988) reported strong seasonality of the effect of leaf soaks of several sand pine scrub plants on the germination and growth of native sandhill grasses. They found that inhibition was concentrated in the late spring and summer, at the onset of the summer rainy season.

In the present study, variation in inhibition observed in soils collected on different sampling dates, as measured by the date and soil \times date interaction terms of the analysis of variance model (Table 3), was not significant for bahiagrass germination but was for bahiagrass dry weight. There was a significant effect of sampling date for little bluestem germination and growth only in the November–April samples. There are no striking seasonal trends to this variation. For example, at some sampling dates, growth of bahiagrass was reduced in *Polygonella* soil compared to both the bare zone and grassed areas, with growth sometimes greater in the bare zone than the grassed area soil (see December 7 and 21, April 26, May 10, and June 21 in Figure 3). At other sampling dates, growth followed the average annual pattern of reduced growth in *Polygonella* soils and intermediate reductions in the bare zone soil (for example, November 23, January 4, June 10, and August 5). Regression analyses did not suggest a correlation of the amount of rainfall in the two weeks preceding each sampling date with grass germination and growth (data not shown). It was noted, however, that significant inhibition in *Polygonella* soil compared to grassed area soils occurred in a greater proportion of samples collected during the summer rainy season of June, July, and August. For little bluestem dry weight, the proportion of sampling dates on which significant inhibition in *Polygonella* soil occurred was significantly higher ($P = 0.03$, Fisher's exact test) in the summer months (five of seven sampling dates) compared to nonsummer months (four of 18 sampling dates). Similar trends are found with bahiagrass germination (inhibition five of seven sampling dates in summer, six of 18 in nonsummer months) and dry weight (inhibition six of seven sampling dates in summer, 11 of 18 in nonsummer months). There does appear, therefore, to be some evidence of a weak seasonal pattern in the inhibition of grass germination and growth.

It should be noted that because soils were assayed under similar environmental conditions, these bioassays measured seasonal variation in the presence and abundance of putative *Polygonella* phytotoxins. This is not necessarily the same as seasonal variation of phytotoxicity under field conditions. The effects of phytotoxins are known to be intensified by environmental stresses such as high temperature and nutrient limitation (Stowe and Osborn, 1980; Einhellig and Eckrich, 1984; Einhellig, 1987); therefore, the actual effects of equal soil concentrations of a phytotoxin may be quite different depending on the time of year. The white sands of the Florida scrub are low in available macronutrients (Table 2; also Kalisz and Stone, 1984), subject to soil surface temperatures in

excess of 50°C on sunny days (Richardson, 1985), and moisture stress is a significant factor in the dry season and during pauses in summer rains. It is likely that these environmental factors contribute to enhanced effectiveness of allelochemicals produced by *Polygonella* and other scrub perennials.

Soil Nutrient Analyses. Unlike the Lake Wales site, there was a higher clay content in the grassed area soils at the Lake Arbuckle scrub, which was the result of road construction. At the edges of the grassed area where samples were taken, these differences were slight (Table 2). Bare zone soil at the Lake Arbuckle site was lower in P, K, Mg, organic matter, and total N than the *Polygonella* and adjacent grassed area soils. *Polygonella* soil was slightly lower in P and K than the adjacent grassed area soils (Table 2). Levels of total and ammonium N were lower in *Polygonella* than grassed area soils (Table 2). Significantly, growth of bahiagrass and little bluestem was greater in bare zone soil than in *Polygonella* soil, which has higher nutrient levels. The reduced growth in *Polygonella* soil relative to the bare zone is thus attributed to the presence of higher concentrations of allelochemicals. The reduced growth in *Polygonella* soil relative to adjacent grassed area soils is attributed to the combined effect of phytotoxins and lower levels of P, K, and particularly N in the *Polygonella* soil. Seed germination is largely unaffected by soil nutrient levels, although it can be stimulated by relatively high concentrations (1–10 mM) of potassium nitrate (Mayer and Poljakoff-Mayber, 1982; Popay and Roberts, 1970). Given the low nitrate levels in these soils, the reduced germination of bahiagrass in the *Polygonella* and bare zone soils is attributed to *Polygonella* allelochemicals.

In summary, results of these studies provide strong evidence in support of the hypothesis that allelochemicals produced by *Polygonella myriophylla* can reduce the germination and growth of other species. Definitive proof awaits characterization of the putative inhibitors and evidence that they persist at concentrations sufficient to produce the observed effects. The involvement of disease organisms or subtle mycorrhizal effects cannot be ruled out, although an examination of *Polygonella*, bare zone, and grassed area soils at the Lake Wales site for mycorrhizal spores did not indicate any substantial differences in the amount or types of spores present. The reduced nutrient levels in the bare zone, along with allelochemical inhibition, may be important in reducing invasion and establishment by other species. An investigation of the phytochemistry of *Polygonella* has revealed the presence of high concentrations of several phenolic compounds in *Polygonella* foliage (Weidenhamer, 1987). The identification of these compounds and their quantification in leaf wash and soils is the subject of continuing work and will be reported separately.

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ODOR PREFERENCES OF WILD STOCK FEMALE
HOUSE MICE (*Mus domesticus*) TESTED AT
THREE AGES USING URINE AND OTHER
CUES FROM CONSPECIFIC MALES
AND FEMALES

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Abstract—Urinary chemosignals from conspecific males and females can influence the physiological processes of sexual development in female house mice. The experiments reported here involved testing the odor and whole animal preferences of female mice presented with stimuli from male mice and grouped female mice. Mice were tested at three different ages. Five types of tests were performed: an olfactometer test using whole animals as stimuli, a second olfactometer test using urine stimuli, a soiled bedding substrate preference test, an animal association test, and a stick-chewing test. The results from all tests indicate that prepubertal females avoided male odors and prefer odors associated with grouped females. By about the age of puberty, the females exhibited a preference for male cues and generally avoided cues from grouped females. As adults, the females strongly preferred cues associated with males and avoided cues from grouped females. Thus, female mice appear to be exhibiting some control over their own physiological development and their reproductive condition by the nature of the stimuli with which they associate. Their stimulus preferences may have consequences for their fitness.

Key Words—House mouse, *Mus domesticus*, female odor preference, age effects, conspecific odors.

INTRODUCTION

Treating female mice with urine from male mice, female mice in estrus, and female mice that are pregnant or lactating all accelerate the onset on first vaginal estrus (Vandenbergh, 1969; Colby and Vandenbergh, 1974; Drickamer and Hoover, 1979; Drickamer, 1986a), whereas treatment with urine from grouped female mice delays the onset of first estrus (Vandenbergh et al., 1972; Drickamer, 1974, 1977). The urinary chemosignals that influence the development of the reproductive system have been explored with respect to a variety of factors pertaining to both the chemosignal donors and the female recipients (Vandenbergh, 1983; Drickamer, 1986b). The long-term consequences of accelerated and delayed puberty in terms of survival and reproductive output have also been investigated. Mice that have accelerated puberty have higher mortality rates and have, on average, smaller litters than females that are delayed in attaining the age of first reproduction (Drickamer, 1988).

Previous work on house mice indicates that they are capable of using olfactory cues, particularly those in urine, to recognize local population vs. strange population odors (Cox, 1984), kin (Kareem and Barnard, 1986; Winn and Vestal, 1986), sex (Hayashi and Kimura, 1974; Davies and Bellamy, 1974; Winn and Vestal, 1986), stress (Carr et al., 1970), and specific individuals (Bowers and Alexander, 1967).

Until recently, two types of questions regarding urinary chemosignals influencing reproductive physiology and the onset of puberty had not been investigated. These questions pertain to the deposition of the urine containing the chemical signals and the olfactory choices by the mice for urine and related cues from various sources, e.g., adult male mice. In a companion paper to this one (Drickamer, 1989), the patterns of urine deposition are reported. Here investigations are reported of selections by female mice for urine and other cues associated with male and female conspecifics.

METHODS AND MATERIALS

General Methods

The mice used for these experiments were third- and fourth-generation descendents of a stock of wild house mice (*Mus domesticus*) trapped near West Simsbury, Connecticut. All mice were maintained in opaque polypropylene cages measuring 28 × 15 × 15 cm deep with fitted wire lids containing food and water receptacles. A bedding of ground wood shavings was changed once each week. Pregnant female mice were given cotton squares for making nests. Wayne Lab Blox and water were provided ad libitum to all mice throughout the experiments, except for the time periods that mice were in the test apparatus.

All colony cages and the various test apparatuses were housed in the same two rooms throughout the 24 months during which the experiments were conducted. These rooms were maintained at 21–25°C and 30–60% relative humidity on a 12-hr dark–12-hr light daily regimen with overhead fluorescent lights on from 0600 to 1800 hr.

Female mice used as test subjects were produced by mating one adult male and one adult female. A total of 383 young females from 147 litters born to 77 different adult females were tested. These 383 subject females were used in a series of five experiments outlined below; mice were assigned at random to be tested at different ages and in different types of test apparatus with two restrictions: (1) no mouse was tested twice in the same apparatus, and (2) no mouse was used twice at the same age in two different types of tests. Mice were weaned from their parents at 25 days of age and housed in cages with two or three like-sex littermates until tested. They were maintained in these housing conditions until the conclusion of testing at about 110 days of age.

Five different types of experimental tests were performed, designed to assess the responses of the females at various ages to pairs of stimuli from males and grouped females involving different combinations of sensory modalities. Experiment I consisted of an olfactometer in which whole mice were used in the stimulus chambers, providing test females with a composite of volatile odor cues. Experiment II involved using the same olfactometer, but urine stimuli only were tested, providing the mice with volatile cues from urine. Experiment III was a substrate preference test wherein subject females were given choices between samples of soiled bedding, providing them with a variety of urinary and fecal odor cues and permitting them to have direct contact with the stimuli. Experiment IV involved test females associating with live mice as stimuli, with the possibility of visual and limited contact cues as well as olfactory cues. Experiment V was a test of the motivation of the test mice to chew through a barrier of balsa wood sticks to obtain access to cotton containing urine or water.

In each of the five experiments, mice of three different ages were tested: (1) 35–45 days of age (prepubertal); (2) 60–70 days (peripubertal); and (3) 100–110 days of age (adult or postpubertal).

For each age, mice were tested for their responses to six different pairs of stimuli: (1) adult male vs. adult male, (2) grouped adult female vs. grouped adult female, (3) blank vs. blank, (4) adult male vs. blank, (5) grouped adult female vs. blank, and (6) grouped adult female vs. adult male. The first three pairs in each instance were designed as control procedures. The nature of the blank varied with each experiment as outlined below. For each pair of test stimuli, 14 mice were tested. Thus, 84 mice were tested at each age in each type of test.

The data analyses for each age group of each experiment were similar. In every test of every experiment, the subject mouse was given a choice between

two alternatives. Ratios were computed for its selection for one alternative vs. the other alternative. (By the nature of the test procedures, neither the numerator or the denominator in any test could be zero.) Because the data were skewed and exhibited variance heterogeneity, they were transformed as the natural logarithm (Lenington, 1983). Because of the natural logarithm of 1, $\ln(1) = 0$, we would expect ratios with positive values to indicate selections of the alternative represented in the numerator and, conversely, negative ratios would indicate selections of the alternative represented in the denominator. Ratios of about 1 would mean equal selection of both alternatives. These ratios were used in one-way analyses of variance, followed by post hoc testing using Duncan's new multiple-range test with $P \leq 0.02$.

Experiment I. Olfactometer—Whole Mouse

Apparatus. The test apparatus for the first experiment consisted of a Plexiglas olfactometer based on that used by Lenington (1983). The two stimulus chambers consisted of Plexiglas cylinders measuring 9 cm in diameter by 20 cm long. The test chamber for the subject mouse was a Plexiglas cube 14 cm on each side with two side ports. Attached to each side port was a Plexiglas cylinder measuring 9 cm in diameter by 20 cm long. The pair of stimulus chambers was connected to the ports on the test chamber. Tygon tubing was used for all connections. The entire apparatus was dismantled and washed with detergent after each trial. The air flow through the apparatus was maintained at a rate that resulted in a turnover of the air in each stimulus chamber once every 20 sec.

Procedure. As outlined in the General Methods above, mice of three different ages were tested, six different pairs of stimuli were employed, and 14 mice were tested for each age-stimulus pair combination. The blank or control condition consisted of an empty chamber. The actual test procedure began by placing mice into the stimulus chambers, using a coin flip to decide randomly which mouse would be on which side of the apparatus. This coin flip was repeated for each separate test. The stimulus males were adults (ages 100–175 days) caged alone and stimulus females were adults (ages 85–180 days) caged in groups of eight for at least 30 days prior to being used as stimulus animals.

The air flow was turned on and allowed to run for 5 min. The female test mouse was then placed into the central chamber of the olfactometer and given a 5-min acclimation period. If, during the first 3 min, the test mouse had not visited each side port for at least 15 sec, the experimenter removed the wire mesh top of the test chamber and gently forced the mouse to spend 15 sec in each side port. For a mouse to be considered in the side port, either during acclimation or during the test that followed, the animal had to have all four feet and its head in the side tube.

Each test mouse was then given a 10-min test during which time the experimenter recorded the total time spent within the stimulus port on each side, and the total time spent in the central chamber. To be used in further analyses, the mouse must have spent at least 50% of its total test time in one port or the other and at least 15 sec must have been spent in each port during the test. Seventeen mice of various ages failed to meet these criteria; these animals were replaced by additional test subjects. The data were analyzed as described in the General Methods, using a ratio of the total time spent in one port versus total time spent in the other port.

Experiment II. Olfactometer—Urine

Apparatus. The apparatus used for this test was identical to that used in the preceding series of tests.

Procedure. The procedure used for this test was identical to that used for the preceding test, except that the stimuli consisted of 0.2-cc samples of urine placed on cotton located in the stimulus chambers leading to the two choice ports of the olfactometer. Male urine was collected from a battery of 25 individually caged adult males, 95–160 days of age. Female urine was collected from a battery of 40 adult females, 85–140 days of age, housed with eight females per cage for a period of at least 30 days prior to the start of urine collection. Fresh urine of each type was collected each day by holding appropriate mice over Petri dishes and gently squeezing the flanks. Urine was collected from at least 10 mice of each type each day. Separate Petri dishes were used for each urine type. Distilled water placed on cotton was used for the blank treatment. In this test 21 mice failed to spend 50 percent or more of the 10 min test period in one side port or the other and at least 15 sec in each during the test period; these mice were replaced by testing additional subjects. The results were analyzed using a ratio like that calculated for Experiment I.

Experiment III. Substrate Bedding Preference

Apparatus. The test apparatus consisted of a 35-liter aquarium with a Plexiglas partition cemented to the center of the floor. A wire mesh lid on top of the aquarium prevented the mice from escaping.

Procedure. Females of all three ages were tested with this apparatus using the six pairs of stimuli, with 14 mice tested for each age–stimulus pair combination. The test procedure involved first placing 100-cc samples of bedding on the two sides of the aquarium; a coin flip for each separate test was used to randomize placement of the bedding. New bedding samples were used for each test. A battery of 32 adult males (ages 100–170 days) and a battery of 10 cages each containing eight adult females (ages 80–145 days) served as donors of soiled bedding. The control condition consisted of clean bedding. For those

donor cages where bedding was removed, 300 cc of clean bedding was added every third day to ensure adequate bedding for the mice and a continuous supply of soiled bedding for the test procedure.

The test mouse was placed on the Plexiglas partition, and a 10-min test was begun during which the location of the mouse was recorded every 20 sec, providing a total of 30 position records. To be considered as being on one side of the apparatus or the other the mouse had to have all four feet and its nose on that side of the apparatus, and during the course of the test period the mouse must have spent at least one 20-sec interval on each substrate. For all mice, at least 27 of the 30 location records met the first criterion, and all mice met the second criterion. Thus, all were included in the data analyses. A ratio was generated consisting of the number of times the mouse was located on one side versus the number of times it was located on the other side of the apparatus. These ratios were analyzed using the procedure described in the General Methods.

Experiment IV. Association Test

Apparatus. The apparatus used to test whole animal associations consisted of a rectangular wooden box measuring 1 m long \times 50 cm wide \times 50 cm high with a wire mesh lid. The apparatus was divided into two halves by a wooden partition. The partition was broken at the bottom center where a wire cage (of 0.5-cm mesh hardware cloth) measuring 30 cm long \times 10 cm wide \times 8 cm high was located with equal portions extending into each half of the box. The wooden portions of the apparatus were covered with epoxy paint to facilitate washing the entire apparatus after each trial.

Procedure. Females of the same three ages used previously were tested with the six pairs of stimuli used in experiments I–III. Stimulus mice, selected from those maintained for use in experiment I were placed on either side of the main test chamber; a coin flip was used at each trial to determine on which side each stimulus mouse would be placed. The control condition consisted of a blank chamber. The test mouse was placed into the wire mesh alleyway at the center of the apparatus and the wire lids were placed on both the alleyway and the main chamber. A 5-min acclimation period ensued during which the observer made certain that the test mouse entered both sides of the alleyway to be exposed to the stimulus there for at least 15 sec; if the mouse had not been into both sides by the end of 3 min of the acclimation period, the observer carefully opened the main chamber and used a small probe to induce the test mouse to cross over to the other side of the test alleyway for at least 15 sec.

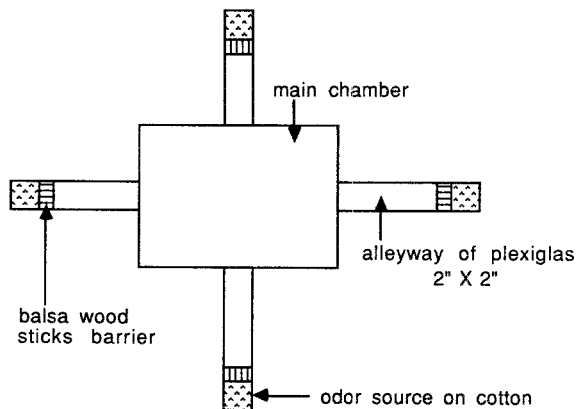
After the 5-min acclimation period, the mouse was observed for a 10-min test period during which the observer recorded the amount of time the mouse spent on each side of the apparatus. To be used in further analyses, the mouse

must have spent at least 30 sec of the total test period on each side of the apparatus. Only three mice failed to meet this criterion; additional test mice were used to replace these subjects. Ratios were generated of the time spent on one side versus the other of the apparatus, and the ratios were subjected to the same types of analyses used in previous experiments.

Experiment V. Stick-Chewing Behavior

Apparatus. The apparatus consisted of a 25-cm² central chamber with four alleyways radiating outward, one from each side of the square (Figure 1a). Each alleyway consisted of a 5-cm² made of Plexiglas (Figure 1b). At the end of each alleyway there were two small Plexiglas cubes; the one nearest to the central chamber contained a series of 14 balsa wood pegs arranged into three rows (5,

(a) overhead view of entire apparatus



(b) side view of alleyway

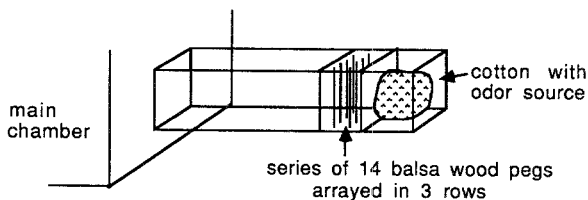


FIG. 1. Stick Chewing Apparatus. (a) A top view of the entire apparatus showing the main chamber where the subject mouse was placed and the possible four side alleyways ending with barriers of balsa wood sticks placed in front of a stimulus chamber with cotton containing urine. (b) A side view of just the alleyway illustrating the barrier of balsa wood sticks and the stimulus chamber.

4, 5), and the outer cube was used to provide urine test stimuli placed on small pieces of cotton. For the present series of tests, only two side alleyways, located on opposite sides of the central chamber were used. The entire apparatus was cleaned with soap and water after each trial.

Procedure. All three ages of test females and the six combinations of stimulus urine sources were used, with 14 mice tested for each age-stimulus pair combination. Fresh urine was collected for each test using the same batteries of donor mice described previously for experiment II. The test mouse was placed into the central chamber and, at the end of 6 hr, the mouse was removed and the numbers of sticks chewed in front of each of the two stimuli were counted. These stick counts were used to generate ratios, and analyses were performed on the ratios as in the other experiments.

RESULTS

For all five experiments at all three ages and the tests involving pairs of stimuli of the same type, there were no significant deviations from the expected value of 1.0. These data are graphed on the left-hand side of each of the five figures that display the results of the experiments.

Experiment I. Olfactometer—Whole Mouse

Prepubertal female mice avoided the whole body odors of males relative to the control condition, exhibited some preference for the odors from a grouped female relative to the blank and showed a marked preference for the odors from grouped females when tested against males ($F = 22.96$; $df = 5,78$; $P < 0.001$) (Figure 2). By 60–70 days of age, the females showed no significant deviations from equal choices in all stimulus pairs ($F = 0.46$; $df = 5,78$; $P = \text{NS}$) (Figure 2). Adult females showed a clear preference for the male odor when it was tested with either the blank or with a grouped female and avoided the odor from a grouped female relative to the blank condition ($F = 16.11$; $df = 5,78$; $P < 0.001$) (Figure 2).

Experiment II. Olfactometer—Urine

Female mice 35–45 days of age avoided male urine when it was paired with water, but preferred male urine compared to urine from grouped females, and exhibited no differential selection when presented with urine from grouped females and water ($F = 3.72$; $df = 5,78$; $P < 0.005$) (Figure 3). Peripubertal females spent more time near the odor of males than either grouped females or water and did not exhibit a selection preference between the blank and the odor

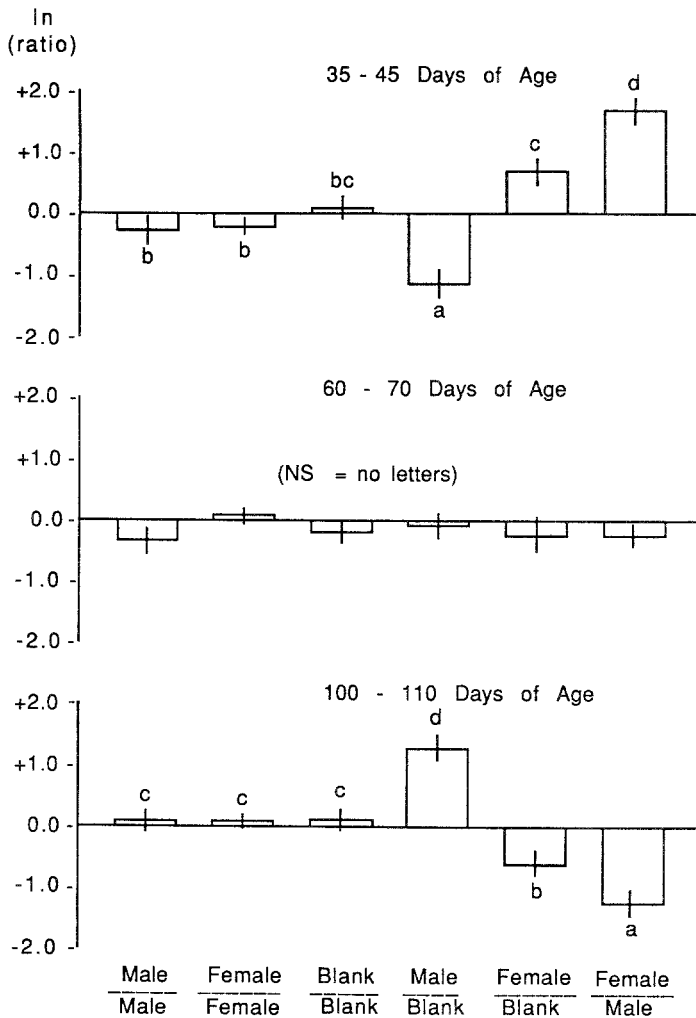


FIG. 2. Means of natural logarithms of ratios of seconds spent on two sides of the olfactometer (± 1 SEM = vertical lines) using live mice as stimuli. Female mice were tested at three different ages. Pairs of live mouse types that were placed into the two stimulus chambers of the olfactometer are indicated at the bottom of the histogram. The blank stimulus condition consisted of an empty chamber. Fourteen mice were tested for each stimulus pair at each age. Within an age group, those bars not marked with the same letter are significantly different at the 0.02 level by Duncan's new multiple-range test.

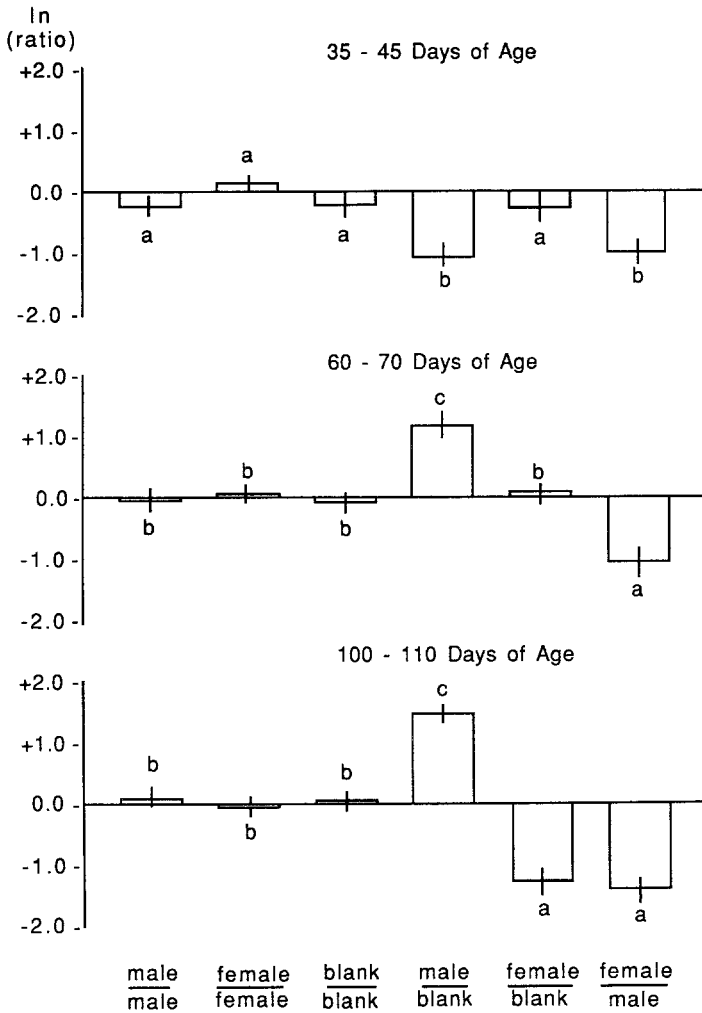


FIG. 3. Means of natural logarithms of ratios of seconds spent on two sides of the olfactometer (± 1 SEM = vertical lines) using mouse urine as stimuli. Female mice were tested at three different ages. Pairs of mouse urine types that were placed into the two stimulus chambers of the olfactometer are indicated at the bottom of the histogram. The blank stimulus condition consisted of water placed on the cotton. Fourteen mice were tested for each stimulus pair at each age. Within an age group, those bars not marked with the same letter are significantly different at the 0.02 level by Duncan's new multiple-range test.

of grouped females ($F = 11.51$; $df = 5,78$; $P < 0.01$) (Figure 3). Adult females preferred male odor to the water or the urine of grouped females, and they avoided the urine odors of grouped females when paired with water ($F = 22.02$; $df = 5,78$; $P < 0.001$) (Figure 3).

Experiment III. Substrate Bedding Preference

Prepubertal females chose the female-soiled bedding over the clean bedding or bedding soiled by males and avoided male-soiled bedding when it was paired with clean bedding ($F = 19.96$; $df = 5,78$; $P < 0.001$) (Figure 4). Females 60–70 days old preferred male-soiled bedding over clean bedding and over bedding soiled by grouped females and did not exhibit a selection preference between clean bedding and bedding soiled by grouped females ($F = 15.17$; $df = 5,78$; $P < 0.001$) (Figure 4). Adult females preferred soiled bedding when tested against either clean bedding or bedding soiled by grouped females, and they preferred clean bedding when tested with bedding soiled by grouped females ($F = 25.09$; $df = 5,78$; $P < 0.001$) (Figure 4).

Experiment IV. Association Test

Females 35–45 days old spent more time in association with the empty chamber or with the grouped female than with the male but did not exhibit a selection preference when tested with an empty chamber and a grouped female ($F = 25.173$; $df = 5,78$; $P < 0.001$) (Figure 5). At age 60–70 days, the females associated more with the male than with a grouped female, and more with the male than with a blank chamber, although this last mean was not different from that for the test involving a blank chamber vs. a grouped female, and the latter, in turn, was not different from the control values ($F = 6.791$; $df = 5,78$; $P < 0.005$) (Figure 5). Adult females spent more time in association with the male when that stimulus was paired with either the blank chamber or a grouped female, and they spent more time with the empty chamber than with the grouped female ($F = 27.997$; $df = 5,78$; $P < 0.001$) (Figure 5).

Experiment V. Stick-Chewing Behavior

Prepubertal females chewed through more sticks in front of the urine from grouped females than cotton with water, but chewed through more sticks with the water than male urine; they did not differentially chew through sticks when the test pair consisted of grouped female urine paired with water ($F = 13.08$; $df = 5,78$; $P < 0.001$) (Figure 6). Females 60–70 days old chewed through more sticks in front of the male urine than in front of grouped female urine but did not exhibit a selection preference in the other test pairs ($F = 7.42$; $df = 5,78$; $P < 0.001$) (Figure 6). Adult females chewed through more sticks to

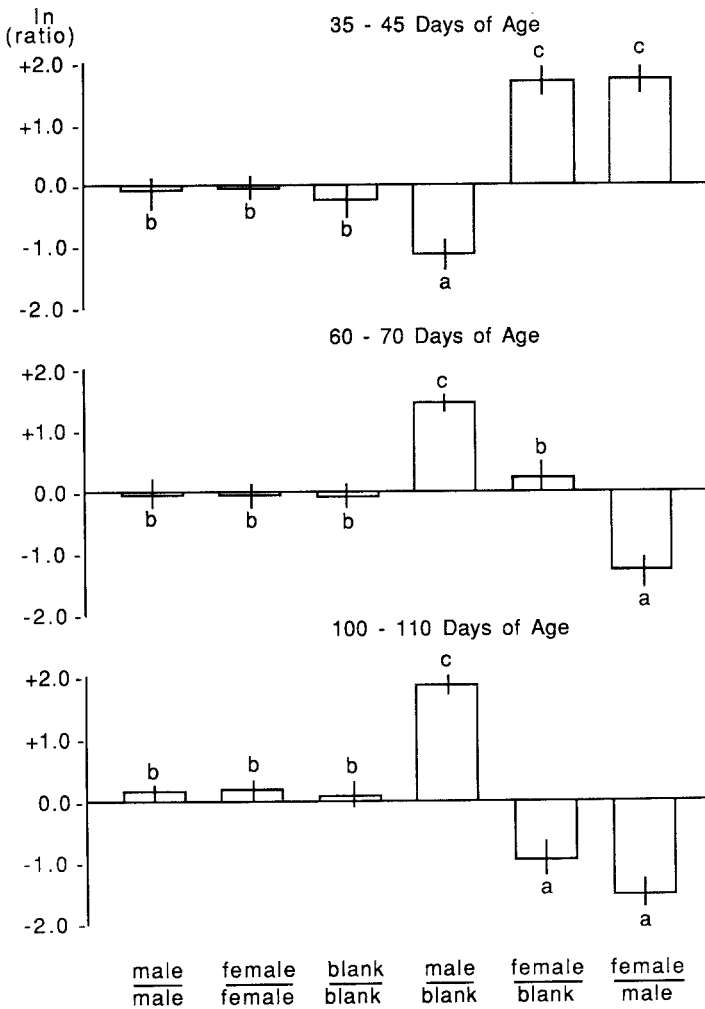


FIG. 4. Means of natural logarithms of ratios of seconds spent on two sides of the substrate preference apparatus (± 1 SEM = vertical lines) using soiled mouse bedding as stimuli. Female mice were tested at three different ages. Pairs of mouse bedding types that were placed into the two sides of the aquarium are indicated at the bottom of the histogram. The blank stimulus condition consisted of clean bedding. Fourteen mice were tested for each stimulus pair at each age. Within an age group, those bars not marked with the same letter are significantly different at the 0.02 level by Duncan's new multiple-range test.

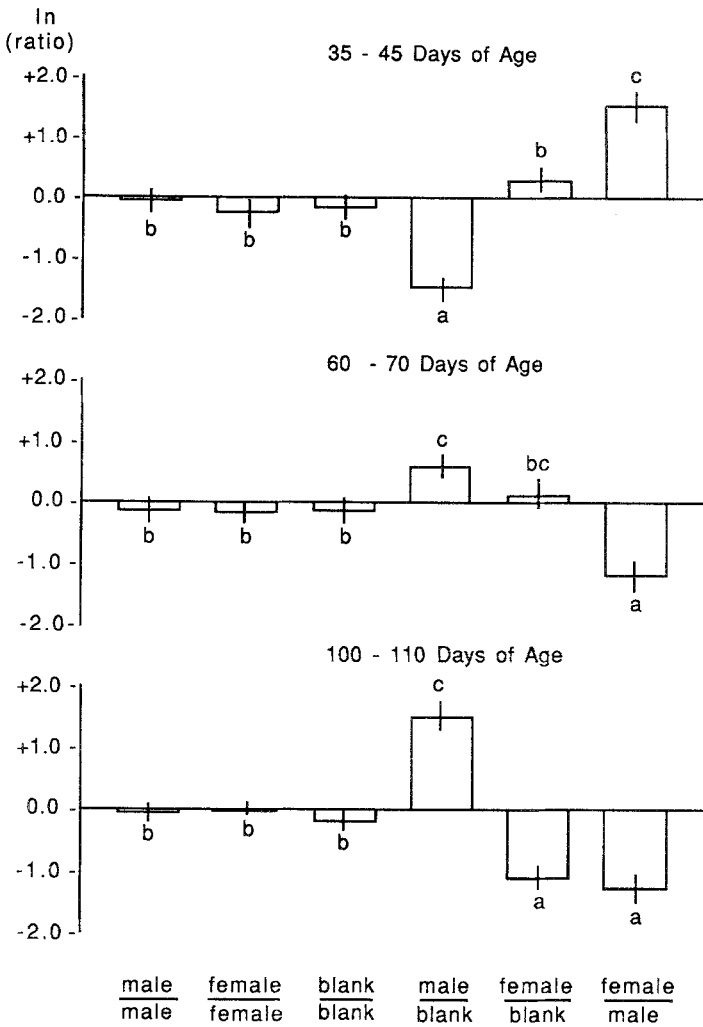


FIG. 5. Means of natural logarithms of ratios of seconds spent on two sides of the whole animal association apparatus (± 1 SEM = vertical lines) using live mice as stimuli. Female mice were tested at three different ages. Pairs of live mouse types that were placed into the two sides of the apparatus are indicated at the bottom of the histogram. The blank stimulus condition consisted of an empty chamber. Fourteen mice were tested for each stimulus pair at each age. Within an age group, those bars not marked with the same letter are significantly different at the 0.02 level by Duncan's new multiple-range test.

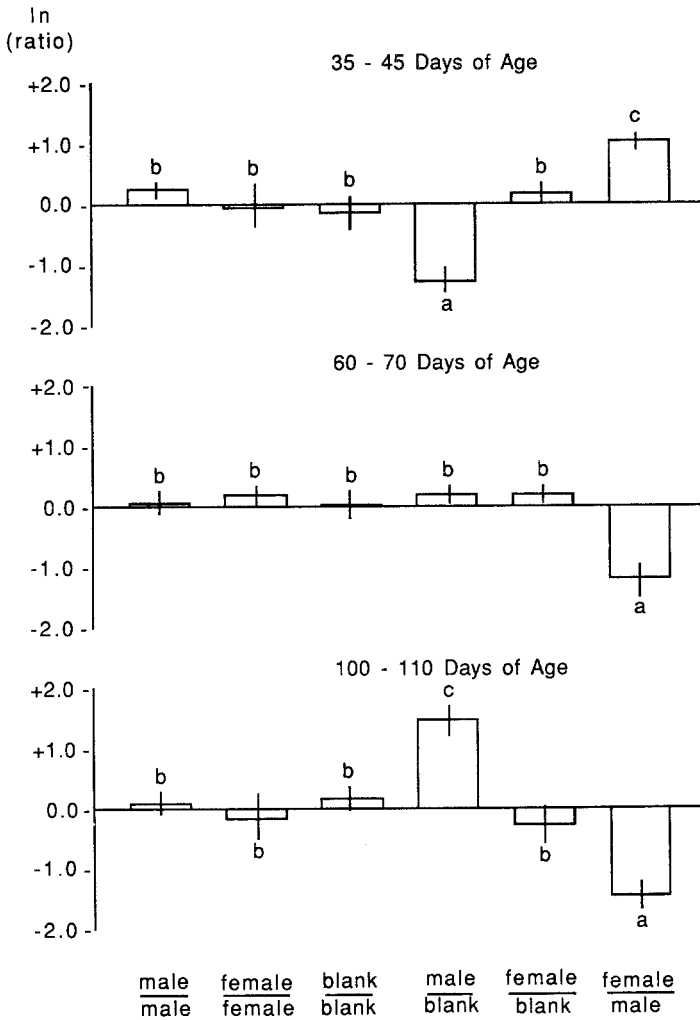


FIG. 6. Means of natural logarithms of ratios of the number of sticks chewed through at two alleyways of the stick-chewing apparatus (± 1 SEM = vertical lines) using mouse urine as stimuli. Female mice were tested at three different ages. Pairs of mouse urine types that were placed onto the cotton in the two alleyways are indicated at the bottom of the histogram. The blank stimulus condition consisted of an empty stimulus chamber at the end of an alleyway. Fourteen mice were tested for each stimulus pair at each age. Within an age group, those bars not marked with the same letter are significantly different at the 0.02 level by Duncan's new multiple-range test.

obtain access to cotton with male urine than either cotton with water or cotton with urine from grouped females and did not exhibit differential stick chewing when tested with urine from grouped females and water ($F = 14.50$; $df = 5,78$; $P < 0.001$) (Figure 6).

DISCUSSION

The foregoing series of five experiments supports the following three major conclusions: (1) Female mice of all three age classes tested exhibit differential selection of cues associated with male and grouped female conspecifics. (2) The differential selection is generally consistent within an age class, regardless of which cues are made available to the mice for use in making their selection. (3) The selections change considerably with the age of the female.

The first conclusion is supported by data from all but one of the analyses. I do not have any explanation to offer as to why there were no significant differences when females 60–70 days old were tested using whole mice as stimuli in the olfactometer apparatus. The lack of differences may be the result of the behavior of the mice; based upon the cues present, female mice of this age either cannot or do not exhibit any selection. Alternatively, the lack of differences may be the result of a statistical probability that out of 15 tests, at least one might be expected to fail to show any significant results. I also do not have any explanation to offer for the seemingly anomalous behavior of prepubertal mice tested with urine in the olfactometer. That these mice exhibited a selection for male urine over that of grouped females is not consistent with the results of the other four tests. Further testing would be required to substantiate and pursue further this potentially interesting result.

With the exception just noted, the patterns of preferences were quite similar across all five types of test apparatus used. The female mice are capable of using a variety of cues, either from a single modality or multiple modalities, to discriminate among the two stimuli presented in any given test. This may be significant in terms of the behavior of the mice; they can apparently respond to any of a variety of stimuli that signal the presence of a particular type of conspecific. One suggested explanation for these findings is, as suggested recently (Drickamer, 1989), that female mice are exhibiting some degree of control over their own reproductive physiology by the odor and related social cues with which they associate. If that is true, then the ability to make the selections based upon volatile body odors, volatile urine odors, nonvolatile odors in soiled bedding etc., could enhance the capacity for influencing their own physiology, including puberty, pregnancy, etc. An equally valid alternative explanation involves possible changes in the motivational state of the female with age. Young female mice may not be sexually motivated; their motivations may be

more directed toward familiar cues associated with maternal care, e.g., food, warmth. At about the time of puberty this pattern may change, resulting in increasing sexual motivation and attempts to locate potential mates. Further study of the behavior of the females and a better understanding of the internal changes associated with the observed behavior patterns will be necessary to distinguish between these alternative, although not mutually exclusive, explanations.

It is also noteworthy that in the final experiment the females not only exhibited selections similar to those observed in the general patterns from the other four tests, but in this last instance they were given the opportunity to work to gain access to the urine odor cues. They did so with the same selection pattern as the other four tests.

The last conclusion may be the most significant. As young mice, females avoided cues associated with male mice, generally preferring to associate with cues from grouped females. In most cases, females that were at about the age of puberty showed a shift in selection, being found more often near or in association with cues from males and either exhibiting an avoidance of, or no clear selection for, cues associated with grouped females. As adults, the females select male cues and avoid cues from grouped females. As has been shown recently (Drickamer, 1988), female mice that attain puberty at an early age, through the effects of either male urine or the presence of a male, have lower survival rates, smaller litters, and fewer pups in their lifetimes than females that are delayed in attaining puberty and/or first mating. Thus, there may be an explanation of the behavior of prepubertal females; they avoid male cues until after attaining puberty. Bronson (1979) proposed that females did not reach puberty until after dispersal, a suggestion that is consonant with the present findings and interpretations. Mice that avoid cues associated with males prior to puberty and until after dispersal would be effectively avoiding possible incestual matings with the father or male siblings and would also be ensuring a possible longer survival and greater reproductive output. Additional work will be needed to determine whether the higher mortality in females that commence reproduction very early is due to insufficient morphological development to properly maintain the pregnancy, other side effects from the early pregnancy, or factors not yet identified.

Beginning at about the time of puberty, female mice apparently seek out cues associated with males; the males represent potential mates. Given the short average lifespan of house mice (Bellamy, 1981), they must seek out potential mates at young ages, although not, apparently, until after the risks associated with early maturation have passed.

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TOXICOKINETICS OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE (DIMBOA) IN THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (HÜBNER)

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Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the major hydroxamic acid present in corn, and its tritiated derivative, were prepared synthetically for use in the determination of the toxicokinetics of this insect deterrent in the European corn borer (ECB), *Ostrinia nubilalis*. In growth studies with DIMBOA (0, 0.05, 0.2, and 0.5 mg/g diet), the mean time to pupation and adult emergence were significantly lengthened by an increase in concentration. Pupal and adult weights, for both female and male, decreased with an increase in concentration. Increased larval and pupal mortality occurred at the highest concentration of DIMBOA. DIMBOA, at concentrations of 0.2 and 0.5 mg/g diet, resulted in a decrease in the number of egg masses produced per female, and at 0.5 mg/g diet, in a decrease in the number of eggs per egg mass. Larvae fed from the neonate stage on a diet containing 0.2 mg [³H]- + [¹H]DIMBOA/g diet showed an increase in the content of label from fourth to fifth instar, but levels declined at pupation and emergence. A large amount of the labeled compounds was excreted by the insect in the pupal case. In dose-related studies, both uptake and excretion increased with an increase in concentration of DIMBOA (0.05, 0.2, 0.4 mg/g diet), while a body burden (concentration in the tissues/concentration in the frass) of approximately 0.25 was maintained for all concentrations. At the highest dose of DIMBOA (0.4 mg/g), the ECB increased consumption, possibly to compensate for the toxic effects of the compound. In topical application studies, elimination of the labeled compound in the frass was rapid, reaching 65% by 4 hr and 88% by 48 hr. Accumulation of label in tissues other than hemolymph was low. The results show that the ECB does possess adaptive mechanisms to deal with the effects of this host-derived compound.

Key Words—European corn borer, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, corn, DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, tritiated DIMBOA, toxicokinetics, topical application, body burden, growth.

INTRODUCTION

Development of resistant lines of corn is one of the principal methods employed to control the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera; Pyralidae), a major pest of corn in North America. Resistance to first brood leaf feeding has a phytochemical basis and has been found to be highly correlated with the level of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the major hydroxamic acid present in corn (Klun and Brindley, 1966; Klun et al., 1970; Reed et al., 1972; Robinson et al., 1982; Zuñiga et al., 1983). DIMBOA is present in the uninjured corn tissue in the glucoside form, and only when the tissue is damaged is it converted to DIMBOA by the hydrolytic enzyme β -glucosidase. Subsequently, DIMBOA decomposes in a reaction that is pH and temperature dependent (Woodward et al., 1978; Perez and Niemeyer, 1986), to yield 6-MBOA (6-methoxybenzoxazolinone) and formic acid.

The level of DIMBOA in the corn varies with age, being highest in the leaves of the embryonic plant and 15-cm seedling (Klun and Robinson, 1969), giving most varieties of corn a high level of resistance at this stage. Levels of up to 4.1 mg DIMBOA/g dry tissue weight have been reported for seedlings (Guthrie et al., 1986). Although DIMBOA is biosynthesized and accumulated throughout the growth of the plant, the concentration decreases with plant height through dilution in growth (Klun and Robinson, 1969; Zuñiga et al., 1983). At a height of about 76 cm, the different cultivars of corn can be segregated into those with high, intermediate, and low concentrations of DIMBOA.

When ECB are fed diets containing DIMBOA at concentrations corresponding to levels in resistant cultivars, reduced larval development and weight at pupation, as well as reduced reproductive capability of the adult, which translates into a smaller number of egg masses, are observed (Reed et al., 1972; Robinson et al., 1982). MBOA has also been shown to have similar biological effects on the ECB, but these occur at much higher concentrations (Klun and Brindley, 1966; Argandoña et al., 1980; Campos et al., 1988).

The toxicokinetics of both of these compounds was examined to gain a greater understanding of the mode of action of DIMBOA and MBOA, and the insect's response to these toxicants derived from the host plant. Our work on MBOA is already published (Campos et al., 1988), and the present report examines in detail the fate of labeled DIMBOA in feeding trials and topical applications in the insect.

METHODS AND MATERIALS

ECB Rearing. The bivoltine strain was originally obtained from the Agriculture Canada Research Station (London, Ontario). The culture was maintained in our laboratory according to procedures and techniques of Guthrie et al. (1972). Diets used in experiments lacked corn cob grits so as to maintain textural uniformity. Compounds were added to the hot diet (55°C) before the agar solidified. All diets containing DIMBOA were changed every three days to ensure that the larvae were exposed to DIMBOA and not its degradation product, MBOA. Analysis of the persistence of DIMBOA in the diet were performed on diet containing a concentration of 5.0 mg DIMBOA/g. The diet cubes were placed in the incubator, under the same conditions as when presented to the larvae, and sampled at 0, 1, 4, 6, and 8 days. The diet cubes were extracted in methanol and DIMBOA content measured using the FeCl₃ method (Woodward et al., 1978). These results showed that approximately 70% of the DIMBOA remained in the diet after three days.

DIMBOA Synthesis. [³H]DIMBOA was synthesized (with some modifications) by the method of Jernow and Rosen (1975). The starting material, 5-methoxy-2-nitrophenol was tritiated using a dilute acid method (Werstiuk and Timmins, 1981) to a specific activity of 0.413 mCi/mmol. The tritiated hydrogens were located on the benzyl ring. The [³H]DIMBOA produced was a pink-tan solid, mp 160°C (rapid decomposition to a dark red tar). The specific activity of the [³H]DIMBOA was 0.443 mCi/mmol. [¹H]NMR (acetone-d₆, 300 MHz) δ 2.85 (bs, OH, D₂O exchangeable) 3.77 (s, 3H, 7-CH₃O) 5.72 (s, 1H, C-2) 6.61 (d, 1H, *J* = 2.6 Hz, C-8) 6.68 (q, 1H, δ_a = 6.70, δ_b = 6.67, *J*_{ab} = 8.8 Hz, C-6) 7.25 (d, 1H, *J* = 8.8 Hz, C-5). [¹³C]NMR (acetone-d₆, 80 MHz) δ 51.0 (CH₃, 7-CH₃O) 88.1 (CH, C-2) 99.2 (CH, C-8) 103.6 (CH, C-6) 109.9 (CH, C-5) 117.8 (C, C-10) 137.7 (C, C-9) 152.5 (C, 153.4). IR (KBr) cm⁻¹ 3345 (br, OH) 3140 (br, Ar-H) 2940 (sh) 1665 (CO), 1605 (aromatic).

Complete synthetic details for DIMBOA and a variety of analogs will be published in the near future as part of a structure-activity investigation.

Growth Studies. Larvae were reared from the neonate stage on diets containing 0, 0.05, 0.2, and 0.5 mg DIMBOA/g diet. Seventy 8-day-old larvae (second instar) from the above diets were placed individually in vials plugged with cotton and provided with an appropriate diet cube and growth parameters measured.

Verification of [³H]DIMBOA as Tracer for DIMBOA. The doses used to determine if the uptake of the tritiated material by the insect was representative of the unlabeled DIMBOA in the diet and topical solutions were: 0.05, 0.10, 0.15, and 0.20 mg [³H]DIMBOA/g diet, to which sufficient unlabeled DIMBOA was added to bring the total concentration to 0.20 mg DIMBOA/g. Fourth-

instar larvae (five replicates with five larvae each) were fed on the above diets for a period of four days. To determine the total content of tritium, larvae and frass were recovered and digested using the tissue solubilizer Soluene-350 (Packard) with Hionic-Fluor (Packard) as the fluorescent medium. ^3H content was measured using a Packard 2000 CA Tri-Carb liquid scintillation counter.

Topical Application. In the topical application study, early fifth-instar larvae (five replicates with five larvae each) were topically applied on the dorsal area behind the head with [^3H]DIMBOA (83.3 ng/mg insect) dissolved in 5 μl of dimethyl sulfoxide (DMSO). The sampling times were 1, 2, 4, 6, 12, 24, and 48 hr. Levels of tritium were measured as described above. The method for the dissection of the larvae has been described in Campos et al. (1988). The concentration of DIMBOA chosen for this experiment did not produce any biological effects on the larvae.

Dose-Related Uptake and Excretion of DIMBOA. The concentrations of DIMBOA in the diets were determined from the growth studies. The concentrations chosen produced significant growth effects in the ECB with respect to controls, but they did not result in high mortality. The concentrations used in these experiments were: 0.05, 0.2, and 0.4 mg DIMBOA/g diet. All diets contained the same level of [^3H]DIMBOA (0.01 mg/g diet); thus a comparison of uptake (consumption) of the labeled parent compound and excretion of labeled compounds can be made as well as a comparison of the uptake of total DIMBOA and excretion of compounds. Larvae were fed from the neonate stage on these diets. The fifth-instar larvae and its frass were collected (five replicates with five larvae each), and the level of tritium was determined as described above. Since the [^3H]DIMBOA was a valid tracer for DIMBOA, the total adjusted radioactivity {counts in frass or insect \times (total DIMBOA/[^3H]DIMBOA)} represents the equivalents of DIMBOA that have passed into insect tissue and frass. Fifth-instar larvae (three replicates of 10 larvae each) fed on the above diets were dissected, as described in Campos et al. (1988), and the distribution of labeled compounds was determined. Larvae fed on the 0.2 mg DIMBOA/g diet were used to determine the accumulation of labeled compounds during the development of the ECB (three replicates of six). Fourth- and fifth-instar larvae were collected as well as pupae, adult, and pupal case.

RESULTS AND DISCUSSION

We have previously observed (Armason et al., 1985; Canney and Gardner, 1988) that the ECB has a greatly reduced antifeedant response to plant chemicals in artificial diets as compared to leaf disk. This phenomenon allowed us to examine the toxic effects of DIMBOA without considering the complication of the effects on feeding behavior on insect performance.

Growth Studies. Data from the growth studies showed that an increase in DIMBOA concentration did not affect the mean weight of the fifth-instar larvae, although it did result in a significant increase in the development time of the larvae (Table 1). Mortality of the larvae, which occurred throughout development, was significantly higher for the larvae on the 0.5 mg DIMBOA/g diet than for those on the control diet. A prolongation of development was not as clearly defined in the pupal instar as in the larval period, although pupal weights were significantly decreased by an increase in concentration (Table 2). Pupal mortality was significantly higher on the 0.5 mg DIMBOA/g diet than for those on the control diet, as was the case for larval mortality.

Total duration of the development of the ECB was increased only at the highest concentration of DIMBOA, for both male and female (Table 3). The weight of adult ECB was significantly decreased by an increase in DIMBOA concentration, especially for females. The presence of DIMBOA did not affect the sex ratio (F/total), indicating that neither of the sexes is more susceptible to the effects of DIMBOA. An increase in DIMBOA, however, did result in a significant decrease in the number of eggs masses produced by females as well as the number of eggs per egg mass (Table 4). Both effects result in a marked decrease in the number of eggs produced by the females. This decrease in fer-

TABLE 1. EFFECT OF DIMBOA (0, 0.05, 0.20, 0.50 mg/g DIET ON DEVELOPMENTAL PARAMETERS OF *Ostrinia nubilalis* LARVAE^a

DIMBOA conc. (mg/g)	Mean wt of fifth instar (g)	Mortality of larvae (%)	Days to pupation
0	0.090a ^b (0.003) (N = 62)	7.5	27.1ab (0.34) (N = 60)
0.05	0.081a (0.003) (N = 33)	8.3	25.84a (0.39) (N = 31)
0.20	0.098a (0.022) (N = 30)	11.8	29.23b (0.72) (N = 27)
0.50	0.072a (0.004) (N = 32)	50.8*	40.29c (1.80) (N = 31)

^aSE in parenthesis; N indicates sample size.

^bMeans followed by the same letter within columns indicate no significant difference ($P \leq 0.05$) in Tukey's Studentized test. Values followed by an asterisk indicate significant difference from the control by the chi-square test ($\alpha = 0.10$).

TABLE 2. EFFECT OF DIMBOA (0, 0.05, 0.20, 0.50 mg/g DIET) ON PUPAL INSTAR OF *Ostrinia nubilalis*^a

DIMBOA conc. (mg/g)	Pupal period (days)		Pupal weight (g)		Pupal mortality (%)
	F	M	F	M	
0	7.85a ^b (0.29) (N = 27)	8.68a (0.35) (N = 19)	0.084a (0.004) (N = 27)	0.074a (0.002) (N = 19)	23.3
0.05	7.73a (0.38) (N = 12)	8.89a (0.35) (N = 13)	0.077a (0.004) (N = 12)	0.061b (0.002) (N = 13)	16.1
0.20	8.13a (0.67) (N = 8)	8.22a (0.60) (N = 14)	0.071ab (0.004) (N = 8)	0.59b (0.002) (N = 14)	18.5
0.50	9.60a (0.51) (N = 9)	9.33a (1.20) (N = 9)	0.054b (0.002) (N = 9)	0.050b (0.002) (N = 9)	40.6*

^aSE in parenthesis; N indicates sample size.

^bMeans followed by the same letter within columns indicate no significant difference ($P \leq 0.05$) in Tukey's Studentized test. Values followed by an asterisk indicate significant difference from the control by the chi-square test ($\alpha = 0.10$).

TABLE 3. EFFECT OF DIMBOA (0, 0.05, 0.20, 0.50 mg/g DIET) ON ADULT STAGE OF *Ostrinia nubilalis*^a

DIMBOA conc. (mg/g)	Days to adult emergence		Mean wt of adult (g)		Sex ratio (F/total)
	F	M	F	M	
0	34.70a ^b (0.48) (N = 27)	34.13a (0.64) (N = 19)	0.050a (0.002) (N = 27)	0.034a (.003) (N = 19)	0.59
0.05	34.08a (0.52) (N = 12)	33.92a (0.60) (N = 13)	0.047ab (0.002) (N = 12)	0.028ab (0.002) (N = 13)	0.48
0.20	36.13a (0.58) (N = 8)	37.08a (0.85) (N = 14)	0.040b (0.003) (N = 8)	0.023b (0.002) (N = 14)	0.36
0.50	45.2b (2.29) (N = 9)	49.67b (2.03) (N = 9)	0.026c (0.003) (N = 9)	0.023b (0.006) (N = 9)	0.50

^aSE in parenthesis; N indicates sample size.

^bMeans followed by the same letter within columns indicate no significant difference ($P \leq 0.05$) in Tukey's Studentized test. A chi-square test ($\alpha = 0.10$) on the sex ratios indicated no significant difference from control.

TABLE 4. EFFECT OF DIMBOA (0, 0.05, 0.20, 0.50 mg/g DIET) ON FERTILITY OF *Ostrinia nubilalis*^a

DIMBOA conc. (mg/g)	Egg masses/F ^b	No. eggs/egg mass	Eggs/F
0	6.7	27.6a (1.0)	179.4
0.05	9.9	21.5a (0.5)	212.9
0.20	0.9*	21.9a (5.9)	19.7
0.50	2.3*	10.0b (0.7)	23.0

^aSE in parenthesis.

^bObtained by placing all emerging males and females in mating chambers. Means followed by the same letter within columns indicate no significant difference ($P \leq 0.05$) in Tukey's Studentized test. Values followed by an asterisk indicate significant difference from the control by the chi-square test ($\alpha = 0.10$).

tility has been observed with ECB fed on varieties of resistant corn (Reed et al., 1972).

Validation of [³H]DIMBOA as Biological Tracer. The amount of ³H isotope found in the fifth-instar larvae and its frass, after feeding on the diets with varying levels of [³H]DIMBOA but the same total level of DIMBOA (³H + ¹H) (0.2 mg/g), was directly related to the amount of ³H isotope in the diet ($P \leq 0.05$, $r = 0.89$, and $r = 0.97$ for the insect and frass samples, respectively) (Figure 1). This linear relation suggests that there was no preferential uptake and excretion of the [³H]DIMBOA over the unlabeled DIMBOA and that the [³H]DIMBOA thus was acting as a reliable tracer for the unlabeled DIMBOA.

Accumulation of Labeled Compound during Development and Dose-Related Uptake and Excretion of DIMBOA. As with MBOA (Campos et al., 1988), the level of label retained by the insect, after feeding from the neonate stage on a diet containing 0.2 mg [³H]DIMBOA/g, increased from fourth to fifth instar but decreased as the insect entered pupal stage and again at emergence. A high level of labeled compounds was excreted in the pupal exuviae at emergence (Figure 2). The level of tritium in the meconium was negligible. The body burden (concentration in the insect/concentration in the frass) at this dose for the fifth-instar larvae is 0.26. These results indicate that the ECB larvae has the ability to excrete a large percentage of the ingested compound and thus minimize the toxic effects of DIMBOA. By further excreting a large percentage of the retained compounds in the pupal exuviae, it minimizes the possible toxic effects to the adult. Metabolism studies have shown that the ECB is able to metabolize both MBOA and DIMBOA (in preparation), thus further minimizing the toxic effects of these compounds.

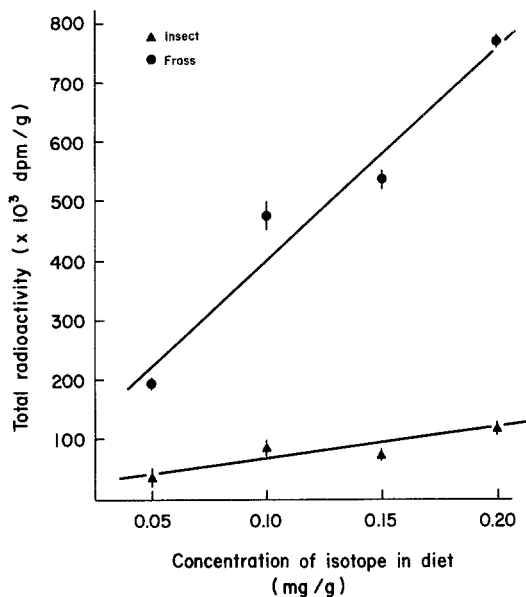


FIG. 1. Relationship between the concentration of labeled DIMBOA in the diet and that found in the fifth-instar larvae (▲) of *Ostrinia nubilalis* and the frass (●). Total DIMBOA was maintained at 0.2 mg/g diet by addition of cold compound. Bars indicate SE

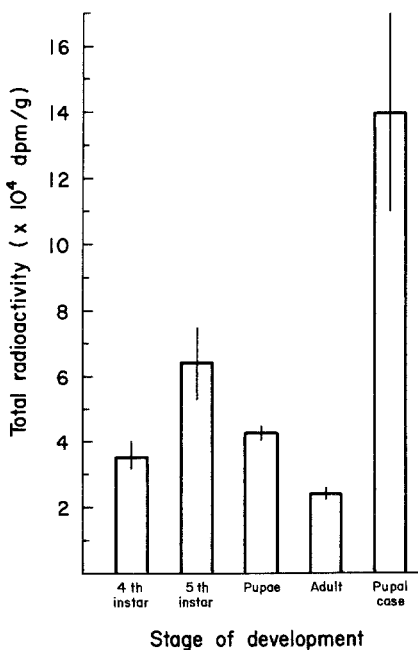


FIG. 2. The level of tritium label in the ECB during its development when fed a diet of 0.2 mg DIMBOA/g diet containing 1.6×10^5 dpm/g diet. Bars indicate SE

A subsequent experiment examined the effect of dietary DIMBOA concentration on the distribution of label in insect and frass. The uptake and excretion of DIMBOA equivalents was found to be affected by concentration (Figure 3). The level of total adjusted DIMBOA (which is a measure of total DIMBOA equivalents that have passed into the insect and frass) increased linearly with an increase in concentration (Figure 3) ($r > 0.95$), resulting in a constant body burden (Figure 3, inset). If only the level of label is considered (Figure 3, inset), at the two lower concentrations used (0.05 and 0.2 mg/g) there was not much difference in the uptake and excretion of label by the insect. At the higher concentration (0.4 mg/g), there was an increase in the accumulation of label in the insect as well as an increase in the level in the frass. One explanation of these results is that the ECB larvae may be consuming more of the diet at the higher concentration than at the two lower concentrations. An increased consumption of food could be attributed to the antinutritional effects of DIMBOA. Argandoña et al. (1982) and Niemeyer et al. (1982) have postulated that the

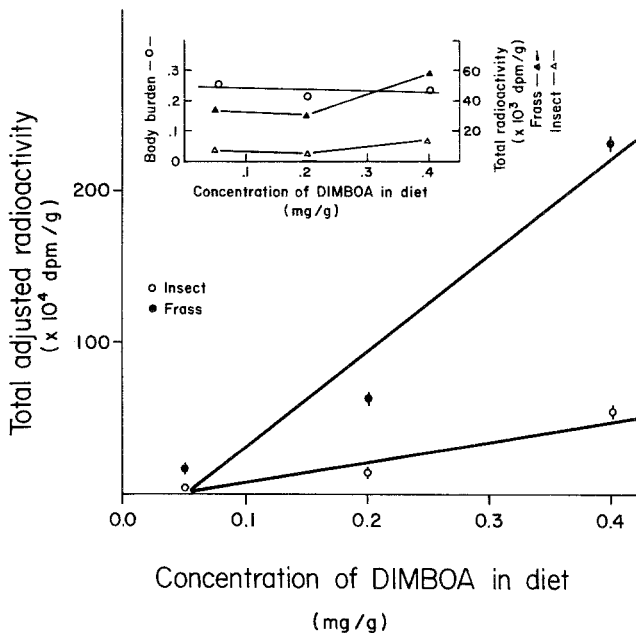


FIG. 3. Dose-related uptake by the fifth-instar larvae of the ECB (○) and excretion in the frass (●) of labeled compounds in feeding trial with DIMBOA. All diets contained the same level of [³H]DIMBOA (4.66×10^4 dpm/g diet), but varying total concentrations of total DIMBOA (³H + ¹H). The total adjusted radioactivity was calculated from counts in frass or insect \times total DIMBOA/[³H]DIMBOA. Inset shows the relationship between concentration of DIMBOA in the diet and body burden (○) and the level of tritium in the insect (△) and frass (▲). Bars indicate SE

toxicity of DIMBOA is due to its ability to bind with enzymes. A reduction in digestive enzyme activity would result in a decrease in available nutrients to the larvae, and the larvae would compensate by increasing their consumption of food. With an increase in consumption, the larvae would be exposed to higher levels of the compound, and the toxic effects would become more noticeable.

Larvae from the above study showed that the ^3H compounds were not sequestered in any specific tissues (Table 5), although the level in the hemolymph (~50%) was higher than in the other body parts looked at. The log P value (the octan-1-ol-water partition coefficient) for DIMBOA [calculated to be approximately 0.8 by the shake flask method (Leo et al., 1971)] shows that this compound is relatively hydrophylic and therefore would not tend to be accumulated in specific tissues. The higher levels of ^3H compounds in the hemolymph indicate that any absorbed DIMBOA, or metabolites, are not sequestered in any specific tissue and are rapidly transported, which is a preliminary to rapid excretion of compounds. MBOA, with a log P value only slightly higher, 1.2 (Campos et al., 1988), shows these same characteristics.

Topical Application. Feeding studies with labeled DIMBOA make it difficult to distinguish between label passing directly through the gut and that which is excreted. To examine more closely the ability of the larvae to deal with the presence of DIMBOA, a topical application study was performed. The results of the topical application of DIMBOA are very similar to the topical application of MBOA (Campos et al., 1988). Absorption by the cuticle is very rapid, as can be seen by the decrease in the methanol rinse of the larvae (Figure 4). Excretion of labeled compounds is very fast. Four hours after application, 65% of the label appears in the frass, and by 48 hr, this has reached 88%. Tissue accumulation is low, as was the case for MBOA. The higher level in the hem-

TABLE 5. EFFECT OF CONCENTRATION ON DISTRIBUTION OF TOTAL RECOVERED ^3H COMPOUNDS IN FIFTH INSTAR OF ECB, *Ostrinia nubilalis*, FROM FEEDING TRIALS WITH DIMBOA^a

DIMBOA conc. (mg/g)	Gut (with contents)	Fat body	Cuticle	Hemolymph
0.05	12.8 (3.0)	13.5 (2.2)	26.2 (3.6)	47.5 (3.9)
0.2	17.9 (1.1)	13.0 (0.7)	20.2 (4.3)	51.1 (3.8)
0.4	15.9 (4.7)	8.4 (1.2)	22.2 (2.2)	53.4 (1.9)

^aLarvae were fed from the neonate stage on these diets. SE in parenthesis. Data expressed as percent of total radioactivity recovered.

olymph 2 hr after application points to the rapid transport and elimination of the compound.

In nature, highly resistant germ plasm may lead to behavior modification that significantly reduces consumption (Robinson et al., 1978). However, most cultivars are not highly resistant, and ECB larvae feed on them, ingest DIMBOA, and are subject to the toxic effects of the compound that are described in our laboratory study. Previous work has also shown that DIMBOA (Reed et al., 1972; Klun et al., 1967; Robinson et al., 1982) and MBOA (Klun and Brindley, 1966, 1980; Campos et al., 1988) are toxic to the ECB. The toxicokinetics of both compounds indicate that the ECB may have some adaptive mechanisms for handling these compounds. DIMBOA and MBOA are rapidly excreted both when topically applied and when placed in the diet. Tissue accumulation is maintained at a very low level, although this may well be a function of the hydrophilicity of the compounds. Further work on the metabolism of these compounds and their effects on digestive enzymes should give a better

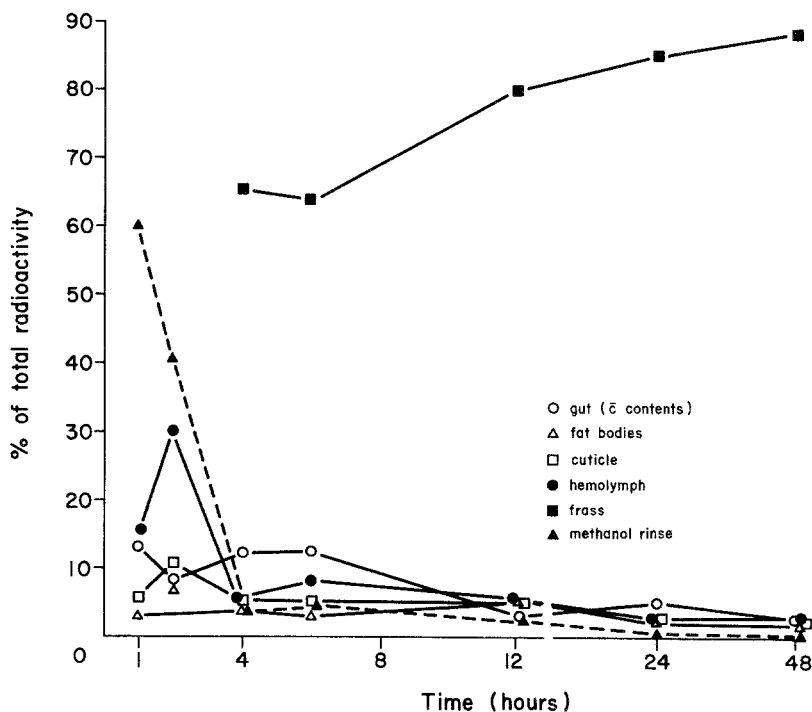


FIG. 4. Toxicokinetics of topically applied $[^3\text{H}]\text{DIMBOA}$ (83.3 ng/mg insect) on the fifth-instar larvae of the ECB, *Ostrinia nubilalis*. Data are expressed as percent of total radioactivity recovered.

indication of the mechanisms of their toxicity and possible adaptive mechanisms the ECB larvae employ to reduce the toxicity.

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DIET-RELATED DIFFERENCES IN THE CUTICULAR LIPIDS OF *Manduca sexta* LARVAE

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Abstract—Cuticular lipid components were examined from fourth-instar larvae of *Manduca sexta* reared on artificial diet or growing plants. The plants used were potato, tobacco, and tomato grown in pots in a greenhouse. Twenty-eight components made up the bulk of the lipids, but there were significant differences in the proportions of them present in insects reared on the different diets. In the case of some insect cuticular lipid components, there was an obvious relationship with the surface components of the plant, but generally this relationship was weak. Nonetheless, the differences may have ecological relevance, as indicated by preliminary work on predation.

Key Words—*Manduca sexta*, Lepidoptera, Sphingidae, insect cuticular lipids, plant cuticular lipids, plant-insect interactions, predator-prey interactions.

INTRODUCTION

The surface lipids of insects have a number of obvious physiological and behavioral roles, especially in relation to regulation of water loss and as a barrier limiting the entry of chemicals and microorganisms (Nelson, 1978; Blomquist and Dillwith, 1985; Lockey, 1988). It is possible that insect surface lipids are in a state of dynamic equilibrium with epidermal cells and that their composition is a reflection of the physiological state of the organism, as has been shown in plants (Cassagne and Lessire, 1975). This may, in turn, have ecological impor-

tance. The present work was initiated because recent results on the predation of caterpillars by ants indicated that prey acceptability was dependent upon the diet of the prey (Bernays, Cornelius, and Espelie, in preparation). Thus artificial-diet-reared larvae of *Manduca sexta* were found to be significantly more palatable to the Argentine ant, *Iridomyrex humilis*, than larvae reared on foliage of certain plant species, although the insect diet was not itself accepted. In addition, acceptability varied among caterpillars fed on different host plants. Ecologically, these differences may have great significance in determining differential mortality on different host plants.

In the course of the studies on Argentine ant, it was found that rejection often occurred after initial contact, suggesting that superficial chemistry of the caterpillars was influencing predator behavior. In preliminary experiments to see whether ants responded to insect cuticular lipids, beakers with or without cuticular lipid extracts of artificial-diet-reared *Manduca* were placed in alternating positions near worker trails of *Iridomyrmex humilis*. Over 5-min periods, there were significantly more ants in the beakers with the larval cuticular lipids than in control beakers with either plain paraffin wax or evaporated solvent only (sign test, $P < 0.01$). Thus, it is possible that predators may use chemical differences on the surface of the larvae as behavioral cues, and the present study is the first investigation of this chemistry.

METHODS AND MATERIALS

Insects. *Manduca sexta* larvae were obtained from a laboratory culture that has been kept on artificial diet for many generations. The artificial diet consisted of 240 g wheat germ, 108 g casein, 96 g sucrose, 36 g Wesson's salt mix, 48 g Torula yeast, 10 g cholesterol, 6 g sorbic acid, 3 g methyl paraben, 0.6 g streptomycin, 12 ml linseed oil, 48 g agar, and 0.4 g vitamin mixture in 2.2 l H₂O. Individuals were either kept on the artificial diet or placed on foliage during the first or second instar. With all foods, growth rates were variable, but individuals used for extraction were early fourth-instar larvae that had fed continuously on artificial diet or one plant species for at least one week at room temperature.

Extractions. Larvae were gently removed from their food source and placed on clean paper towels for 10 min, weighed, placed individually in test tubes (12 × 75 mm), and killed in the freezer at -20°C. Individual larvae were immersed in redistilled hexane for 60 sec at room temperature. A brief hexane extraction was utilized in an effort to remove primarily cuticular lipids and to minimize the extraction of internal components (Blomquist et al., 1987). The hexane was removed, and the larvae were then briefly rinsed with additional

hexane. The hexane extracts from each larva were combined and concentrated under a stream of N_2 .

Analysis. For chemical analysis, extracts were treated with *N,O*-bis(trimethylsilyl)acetamide at 110°C for 10 min. Excess derivatizing reagent was removed under N_2 , and the derivatized extract was resuspended in hexane. Aliquots (usually 1%) were analyzed by combined gas chromatography-mass spectrometry (GC-MS) (Hewlett Packard 5890A/5970). The capillary column (12.5 m cross-linked methyl silicone) was held at 55°C for 3 min after sample injection (splitless), and the oven temperature was then increased to 305°C at a rate of 25°/min and held at this temperature for 8 min. Individual peaks were identified by their mass spectra, which were recorded at 70 eV at intervals of 1.3 sec. Quantitation was based upon integration of total ion chromatograms. The cuticular extracts were analyzed for the presence of wax esters by reduction overnight with $LiAlD_4$ in refluxing tetrahydrofuran followed by $CHCl_3$ versus aqueous extraction and derivatization and analysis by combined GC-MS (Espelie et al., 1983).

The trimethylsilyl ether derivative of a standard sample of cholesterol was found to have identical retention time and mass spectrum to that recovered from the surface of the larvae and was matched by computer search with 40,000 spectra from the National Bureau of Standards Mass Spectral Library. 10-Nonacosanol was recovered from the epicuticular lipids of *Pinus taeda* needles and 4,8,13-duvantriene-1,3-diols from the leaves of *Nicotiana tabacum*. These compounds were converted to trimethylsilyl ethers and utilized as standards for retention time and mass spectral comparison (Holloway et al., 1976; Severson et al., 1984).

Fresh leaves of tomato, potato, and tobacco were dipped in hexane for 60 sec at room temperature, and the extracts were analyzed as described above. Components were identified by their mass spectra (Blomquist et al., 1976; Severson et al., 1984). The foliage extracts were not analyzed for the presence of wax esters. *M. sexta* larvae that had been extracted with hexane were subsequently extracted with redistilled $CHCl_3$ for 1 min at room temperature, and the extracts were derivatized and analyzed as described above.

RESULTS

The surface chemistry of *M. sexta* larvae was found to vary dramatically when the larvae were reared on different food sources (Table 1). All larvae had most of the same *n*-alkanes and methyl-branched alkanes in their cuticular lipids. Although these hydrocarbons were present in approximately equal amounts in the different larval extracts, the proportion that they comprised of the total

TABLE 1. AVERAGE PERCENT COMPOSITION OF CUTICULAR LIPIDS RECOVERED FROM INDIVIDUAL *Manduca sexta* LARVAE REARED ON ARTIFICIAL DIET, OR POTATO, TOMATO, OR TOBACCO FOLIAGE^a

Peak	Component	Diet	Potato	Tomato	Tobacco
1	Hexadecenoic acid	0.0	D ^b	0.0	1.7
2	Hexadecanoic acid	1.2	1.8	1.0	1.9
3	Octadecadienoic acid	3.2	1.7	2.6	0.2
4	α -4,8,13-Duvatriene-1,3-diol	0.0	0.0	0.0	17.7
5	Octadecanoic acid	0.7	1.6	1.2	0.5
6	β -4,8,13-Duvatriene-1,3-diol	0.0	0.0	0.0	28.0
7	<i>n</i> -Tricosane	0.5	0.6	1.1	0.3
8	<i>n</i> -Pentacosane	3.3	4.2	2.3	3.1
9	<i>n</i> -Hexacosane	0.4	0.4	0.3	0.0
10	<i>n</i> -Heptacosane	7.8	12.8	10.9	6.8
11	9,13-Dimethylheptacosane	0.5	1.7	0.2	0.0
12	<i>n</i> -Octacosane	0.3	0.0	0.4	0.0
13	<i>n</i> -Nonacosane	4.9	10.3	7.0	3.9
14	8-, 7-, and 6-Heptacosanol ^c	0.3	2.2	1.9	D
15	Hexacosanol	0.2	0.4	0.1	0.0
16	8,9- and 7,8-Heptacosanediol	D	0.4	0.0	0.0
17	10-, 9-, 8-, and 7-Nonacosanol	0.7	4.2	4.1	D
18	Octacosanol	1.2	1.3	0.3	0.7
19	Cholesterol	20.0	1.3	1.5	0.7
20	9,10- and 8,9-Nonacosanediol	0.1	0.5	0.1	0.0
21	Triacntanol	6.3	0.6	0.2	2.1
22	13,17- and 15,19-Dimethyltrtriacontane	2.1	2.3	2.0	0.0
23	15- and 17-Methylpentatriacontane	3.3	3.2	2.4	2.3
24	13,17- and 15,19-Dimethylpentatriacontane	14.2	20.0	17.1	10.9
25	13,17,21-Trimethylpentatriacontane	1.1	0.8	1.0	0.6
26	13-, 15-, 17-, and 19-Methylheptatriacontane	2.7	4.5	3.4	1.8
27	13,17- and 15,19-Dimethylheptatriacontane	6.2	12.2	9.7	4.7
28	11,15,19-Trimethylheptatriacontane	0.8	0.9	1.1	0.3

^a Components are listed in order of elution. Mean value based on four to six larvae in each case. Unidentified components appeared by mass spectral analysis to be primarily methyl-branched alkanes (approximately 5%) and carbohydrates (5-15%).

^b D: detectable, but less than 0.1%.

^c Elutes with *n*-nonacosane; estimated by integration of selected ion chromatogram.

surface lipids ranged from 35% from larvae reared on tobacco foliage to 76% from those insects reared on potato foliage. The hydrocarbons were dominated by *n*-heptacosane and *n*-nonacosane and by a series of mono-, di-, and trimethyl branched hydrocarbons where the backbone of the chain had an odd number of carbons, with C₃₅ and C₃₇ being the most prominent chain lengths. Several small peaks that eluted between *n*-nonacosane and methylheptatriacontane were

identified as hydrocarbons by their mass spectra, but these components were not present in sufficient quantities to allow complete characterization. The total amount of cuticular lipid recovered by hexane extraction from larvae reared on the same diet varied from one individual to another by as much as sixfold. However, the average amount of cuticular lipid per insect was almost identical for artificial diet and tomato-foilage-reared insects. Those larvae reared on potato foliage and those reared on tobacco foliage had less cuticular lipid (60% and 80%, respectively).

Derivatization of the larval extracts prior to GC-MS analysis allowed identification of components not previously found on the surface of an insect cuticle and the identification of several more components that have been reported only rarely. Variations in the proportion that these components comprised in each extract resulted in the observed diet-dependent differences in larval surface chemistry. The extracts from larvae reared on tobacco foliage were dominated by the diterpenes α - and β -4,8,13-divatriene-1,3-diol, which were identified as their trimethylsilyl ethers (Severson et al., 1984). These diterpenes comprised 46% of the cuticular extract from tobacco-reared larvae but were not found on any of the other larvae (Table 1).

Cuticular lipids from larvae reared on artificial diet contained large amounts of cholesterol and long-chain fatty alcohols, which were identified by the mass spectra of their trimethylsilyl ethers. Free cholesterol comprised 20% of the cuticular extract from artificial-diet-reared larvae, while it equaled only 1–2% of the extracts from larvae reared on the three types of foliage. Free fatty alcohols were approximately 8% of the hexane extract from artificial-diet-reared larvae, but these alcohols totaled only 1–3% of the components from the surface of the other *M. sexta* larvae (Table 1). Triacontanol (C_{30}) was the major fatty alcohol found on the surface of the larvae reared on artificial diet.

Larvae reared on tomato foliage had a high proportion (37%) of methyl-branched hydrocarbons in their cuticular lipids and small amounts of fatty alcohols and cholesterol (1% each). The lipids extracted from the cuticles of these larvae also included C_{29} and C_{27} secondary alcohols. The mass spectra of these components indicated that they were a mixture of positional isomers (Blomquist et al., 1972). The C_{29} secondary alcohols had the hydroxyl moiety on either C_8 (51%), C_9 (33%), C_{10} (10%), or C_7 (6%), while the C_{27} alcohols had the following distribution of isomers: C_7 (52%), C_6 (28%), and C_8 (19%). The amounts of these isomers are estimations based upon the relative intensity of the alpha-cleavage ions generated by cleavage on either side of the derivatized mid-chain hydroxyl. Although positional isomers of the secondary alcohols gave a single peak for each chain length in the total ion chromatograms, selected ion chromatograms (scanning for alpha-cleavage ions) indicated that there had been partial separation with the isomers eluting in decreasing order of carbon substitution number. Integration and comparison of these selected ion chromato-

grams was utilized to estimate the amount of the C_{27} secondary alcohols since they eluted with *n*-nonacosane.

The cuticular lipids of larvae reared on potato foliage were very similar to those isolated from larvae reared on tomato. However, those reared on potato had a higher proportion of methyl-branched hydrocarbons (46 vs. 37%) and slightly higher proportions of primary and secondary fatty alcohols (Table 1). The cuticular extract of potato-reared larvae also had the largest amount (albeit only 1%) of novel components, which appeared, from their mass spectra, to be C_{27} and C_{29} diols. The alpha-cleavage pattern indicated that the larger molecular weight components were a mixture of positional isomers: 8,9-nonacosanediol and 9,10-nonacosanediol (Figure 1). The mass spectra of the shorter chain length homologs indicated that they were 7,8- and 8,9-heptacosanediol.

The cuticular lipid compositions listed in Table 1 are those obtained by a 1-min hexane extraction of the larvae. In an effort to show that the hexane had

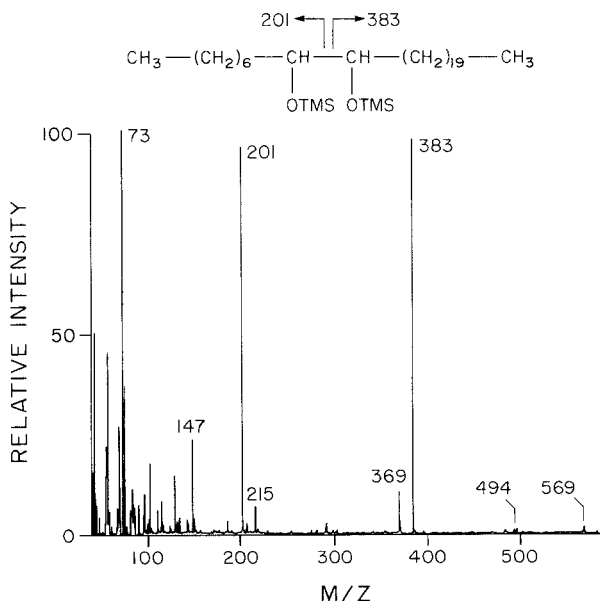


FIG. 1. Mass spectrum of the trimethylsilyl ether of 8,9-nonacosanediol, a minor component in the cuticular lipids of *Manduca sexta* larvae. Dominant ions at m/z 201 and 383 are due to cleavage between the adjacent hydroxyl moieties; the molecular ion is barely visible at m/z 584 and M-15 is seen at m/z 569. Fragments from the positional isomer 9,10-nonacosanediol are seen at m/z 215 and 369. These were the two dominant ions in the spectrum recorded 1.3 sec earlier.

removed most of the cuticular lipids, larvae previously extracted with hexane were immersed in CHCl_3 for 1 min, and the CHCl_3 extracts were derivatized and analyzed by combined GC-MS. With the exception of those larvae reared on tobacco, these extracts had the same cuticular lipid compositions shown in Table 1, but the amount of lipid extract was less than 5% of that obtained in the original hexane treatment. Analysis of the CHCl_3 extracts of hexane-extracted larvae reared on tobacco indicated that the hexane had efficiently removed the cuticular lipids from these larvae, except for the duvatatriene-1,3-diols that were present in the CHCl_3 in the same quantities found in the original hexane extracts.

Since wax esters would not have been detected with the procedure utilized above, the extracts from larvae reared on each of the diets were treated with LiAlD_4 , derivatized and analyzed by GC-MS. There were no changes in the composition or amount of the derivatized fatty alcohols in the total ion chromatograms, indicating that the cuticular lipids of these *Manduca* larvae do not contain significant amounts of wax esters.

Diet-dependent differences in the cuticular lipids were found for each insect examined. However, the values in Table 1 are averages obtained from the analysis of extracts from four to six individual insects reared on each food source. In each case, there were some variations among the components found on individual larvae. Figure 2 shows the total ion chromatograms of the derivatized extracts from larvae reared on artificial diet. Cholesterol (peak 19) was a dominant component in each case, but triacontanol (peak 21) was less than 1% in larvae 1–3 while it comprised 9–20% of the total extracts in larvae 4–6. Octadecadienoic acid (peak 3) ranged from 1% of the cuticular extract in larva 6 to 7% in larva 2 (Figure 2). All of the larval extracts contained unidentified components that eluted between 12 and 13 min. These components, which were identified only as derivatized carbohydrates by mass spectral computer search, comprised 1–5% of the extract from five of the larvae, but they totaled 42% of the total ion chromatogram of the extract from larva 5. Similar variations among individual larvae reared on the same food source were seen in the cuticular extracts from *M. sexta* reared on the three varieties of foliage.

Hexane extracts of tomato, potato, and tobacco foliage were derivatized and analyzed by GC-MS. The total ion chromatograms had very little similarity to those obtained from larvae reared on the respective foliage, with the exception of tobacco where the foliar extract was dominated (47% of total) by the 4,8,13-duvatatriene-1,3-diols as the larval cuticular extracts had been (Figure 3). The remainder of the tobacco leaf extract was a series of hydrocarbons with the major ones being 2-methyltriacontane (8%), 3-methylhentriacontane (7%), 3-methylnonacosane (6%), and *n*-hentriacontane (6%) (Table 2).

Unbranched alkanes were the major components in the hexane extract of

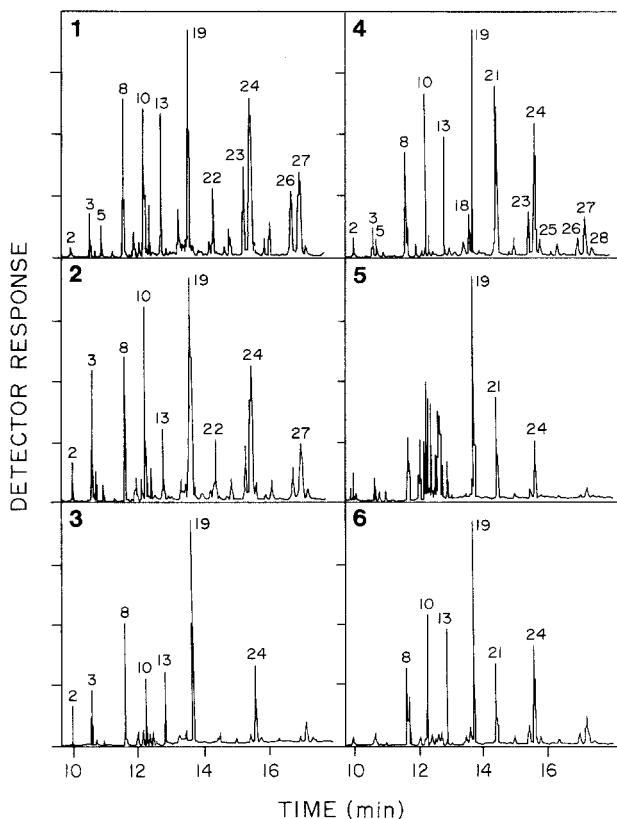


FIG. 2. Total ion chromatograms of the derivatized cuticular lipids isolated from six individual *Manduca sexta* larvae that had been reared on artificial diet. Numbered peaks are identified in Table 1.

tomato foliage. These homologs were *n*-hentriacontane (51%), *n*-tritriacontane (22%), and *n*-nonacosane (5%). Branched hydrocarbons included: 3-methylhentriacontane (9%) and 2-methyltriacontane (4%) (Table 2).

Unbranched alkanes were also the dominant components in the extract of potato foliage: *n*-hentriacontane (38%), *n*-nonacosane (15%), and *n*-heptacosane (12%). Free fatty alcohols were also present in potato leaf surface lipids, but with a different distribution of chain lengths than had been found on the surface of larvae reared on potato: C₂₆ (6% of total extract), C₂₈ (3%), and C₃₀ (0.1%). Fatty alcohols were not detectable in the cuticular extracts from either tomato or tobacco foliage.

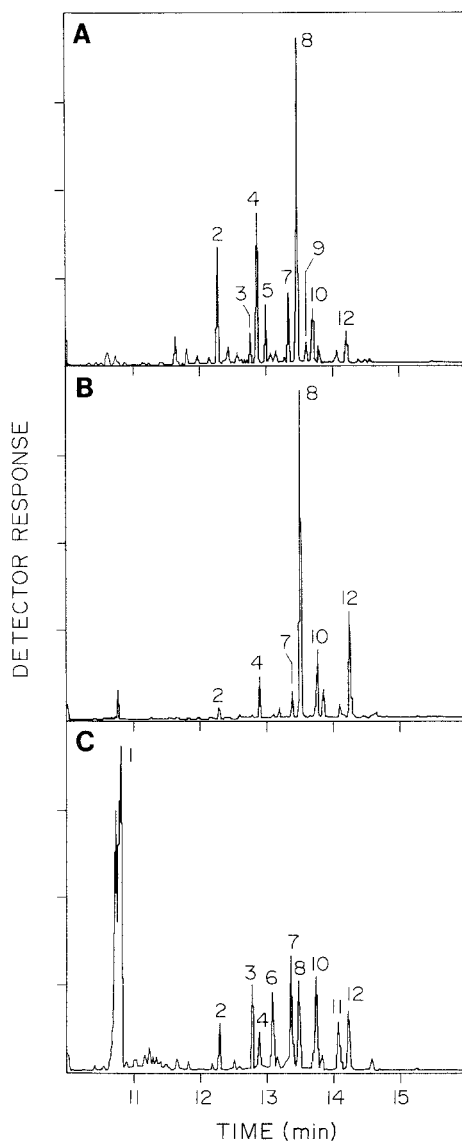


FIG. 3. Total ion chromatograms of the derivatized cuticular lipids isolated by hexane extraction from the foliage of potato (A), tomato (B), and tobacco (C). Numbered peaks are identified in Table 2.

TABLE 2. PERCENT COMPOSITION OF HEXANE EXTRACTS OF POTATO, TOMATO, AND TOBACCO FOLIAGE^a

Peak	Component	Potato	Tomato	Tobacco
1	Duvatrienediols	0.0	0.0	47.0
2	<i>n</i> -Heptacosane	11.7	0.9	1.9
3	2-Methyloctacosane	2.7	0.0	3.7
4	<i>n</i> -Nonacosane	15.4	4.9	3.3
5	Hexacosanol	5.5	0.0	0.0
6	3-Methylnonacosane	0.0	0.0	6.3
7	2-Methyltriacontane	8.7	4.5	8.4
8	<i>n</i> -Hentriacontane	37.6	51.0	6.0
9	Octacosanol	3.2	0.0	0.0
10	3-Methylhentriacontane	6.8	6.0	6.5
11	2-Methyldotriacontane	0.0	0.0	3.7
12	<i>n</i> -Tritriacontane	4.9	22.3	4.3
13	Triacontanol	0.1	0.0	0.0

^aComponents are listed in order of elution.

DISCUSSION

The results clearly show that the surface chemistry of *M. sexta* larvae varies with their food source. The addition of chemicals to the diet has been shown to alter insect surface chemistry (Baker et al., 1978; Brown, 1987), but the rearing of the Mexican bean beetle on different host plants resulted in only minor variations in the cuticular surface chemistry (Bordner et al., 1983; Danehower and Bordner, 1984). This is the first report that variation of the host plant utilized by an herbivore results in dramatic changes in that individual's surface chemistry. Although *n*-alkanes and methyl-branched alkanes were found in each larval cuticular extract, the major homologs were the same in each extract, and they occurred in approximately the same ratio one to another and in approximately the same amounts. The cuticular hydrocarbons of *M. sexta* have been analyzed in detail (Nelson and Sukkestad, 1970; Nelson et al., 1971, 1972), and our results agree very well with these previous reports.

The classes of cuticular compounds that seemed to distinguish which food source a larva had fed upon were the polar components (Table 3). Duvatriene-1,3-diols comprised almost half the cuticular extract from larvae reared on tobacco. Although these diterpenes have not been found on insect cuticles previously, they have been shown to be the dominant component in the cuticle of tobacco leaves (Springer et al., 1975). The duvatrienediols occur on the leaf as sticky globules (Chang and Grunwald, 1980), and some portion of this material

TABLE 3. CUTICULAR LIPID COMPOSITION (%) OF *Manduca sexta* LARVAE REARED ON ARTIFICIAL DIET, OR POTATO, TOMATO, OR TOBACCO FOLIAGE (\pm STANDARD ERRORS)^a

Class of component	Diet	Potato	Tomato	Tobacco
Fatty acids	5.1 \pm 1.8	5.1 \pm 2.1	4.8 \pm 1.3	4.3 \pm 1.5
Duvatrienediols	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	45.7 \pm 5.4
<i>n</i> -Alkanes	17.2 \pm 1.1	30.0 \pm 3.1	22.0 \pm 7.1	14.1 \pm 0.9
Branched alkanes	30.9 \pm 6.0	45.6 \pm 5.0	36.9 \pm 14.1	20.6 \pm 3.1
1° Fatty alcohols	7.7 \pm 3.4	2.3 \pm 0.5	0.6 \pm 0.1	2.8 \pm 2.0
2° Fatty alcohols	1.1 \pm 0.3	7.3 \pm 1.5	6.1 \pm 1.7	D
Cholesterol	20.0 \pm 2.1	1.3 \pm 0.3	1.5 \pm 0.6	0.7 \pm 0.2

^aValues listed in bold face may be classes of compounds that serve to identify the food source of individual larvae.

was probably transferred directly to the surface of the browsing *M. sexta* larvae. Although these polar components would not be uniformly distributed on the insect cuticle, they could still give the larvae unique surface characteristics recognizable by a predator. For instance, spraying duvatrienediols onto the cuticular surface of different plants resulted in oviposition onto those leaves by moths that normally lay their eggs only on tobacco plants (Cutler et al., 1986).

The large amount of free cholesterol (20% of the cuticular lipid) that was found on the surface of *M. sexta* larvae reared on artificial diet (Table 1) may also have been acquired by the insects coming in contact with the food source. Previously, the highest reported levels of free cholesterol in insect cuticle had been 5–6% of the surface lipid of the fleshfly, *Sarcophaga bullata* (Jackson et al., 1974), and of the beetle, *Epilachna varivestis* (Daneshmand and Bordner, 1984). The presence of the long-chain fatty alcohols, which were dominant on some of the *M. sexta* larvae reared on artificial diet, might also result in a characteristic surface. When long-chain fatty alcohols are the dominant cuticular lipid of a plant, the leaf surface often has a characteristic plate morphology due to the crystallization of the alcohols (Jeffree et al., 1975). Two species of saturniid moth larvae, whose cuticular lipid is primarily triacontanol, and a sawfly larva, which has primarily hexacosanol for its cuticular lipid, have a characteristic appearance with the fatty alcohol deposited as a white powder, which may serve to reduce predation (Bowers and Thompson, 1965; Jones et al., 1982; Percy et al., 1983).

Free secondary alcohols comprised 6–7% of the cuticular lipids of potato- and tomato-reared *M. sexta* larvae. Asymmetrical secondary alcohols on the surface of plant cuticles (principally 10-nonacosanol) have been shown by scan-

ning electron microscopy to appear as hollow tube crystals (Holloway et al., 1976). If these secondary alcohols have a similar appearance on the surface of *M. sexta*, they could provide characteristics that would be very distinct to predators. Free secondary alcohols have been found only rarely in insects: as trace components in the cuticle of the grasshoppers, *Melanoplus sanguinipes* and *M. packardii* (Soliday et al., 1974), and as 2% of the cuticular lipids of a weevil, *Ceutorrhynchus assimilis* (Richter and Krain, 1980). The cuticular lipids from the grasshoppers also contained esters of secondary alcohols, with C₂₃ and C₂₅ being the most common chain lengths of both the esterified and free alcohols (Blomquist et al., 1972; Soliday et al., 1974). The hydroxyl moiety was most frequently located on C₁₁ or C₁₂ in both of these secondary alcohols, while the alcohols from *M. sexta* larvae were located on lower-numbered carbons (C₆-C₁₀). The C₂₇ and C₂₉ diols, which are found as minor components on the *M. sexta* larvae, probably originate from the secondary alcohols of the corresponding chain lengths. However, such diols have only been found on the larval cuticle of the beetle *Tenebrio molitor* where 8,9-pentacosanediol comprised 54% of the total lipid (Bursell and Clements, 1967). *M. sexta* is, therefore, the first insect in which long-chain secondary alcohols and diols have been reported to cooccur. Interestingly, the cuticle of *M. sexta* pupae also contains unique oxygenated lipids: oxoalcohols and oxoaldehydes of 26, 27, or 28 carbons that are found either free or esterified (Buckner et al., 1984).

Previous reports have shown a correlation between the chemical composition of the lipids from an insect's cuticle and that of the organic solvent-soluble lipids from the cuticle of its host plant (Blomquist and Jackson, 1973; Hendry et al., 1976; Richter and Krain, 1980). However, the duvatrienediols from tobacco were the only components from the leaf cuticles that were found on the *M. sexta* larval surfaces in proportions similar to those on the plant. *n*-Hentriacontane was the dominant *n*-alkane on all three leaf cuticles, but *n*-heptacosane was the most abundant *n*-alkane on each of the larvae. The 3-methyl- and 2-methylalkanes that were found in the tobacco and tomato leaf cuticles were not observed in the lipid extracts of the larvae that had fed upon either of those plants.

The basis of the variation in cuticular lipid composition among insects reared on the same diet is unknown, although individuals were at different stages in the instar and may have fed on leaves of different ages on the plant. In addition, there is the possibility of interplant variation. The significance of differences between larvae on different diets is unknown. Work is in progress on palatability to ants of larvae from different diets, and we know at present that artificial diet-reared insects are preferred to plant-reared insects (Bernays, Cornelius and Espelie, in preparation). Since the caterpillar diet is unacceptable to ants, we believe that the result is unlikely to be due to surface contamination. It is possible that high cholesterol in the cuticular lipids of diet-reared larvae is

important since this is the largest and most consistent difference from the cuticular lipids of plant-reared larvae. We also know that palatability varies with the host plant, but there seems little basis for any chemical crypsis due to cuticular lipid components among plant-reared larvae since the differences between insect surface lipids and their host cuticular lipids are so great. However, plant waxes are now known to play important roles in herbivore behavior (Bernays et al., 1975, 1976; Chapman, 1977; Woodhead, 1983; Woodhead and Chapman, 1986; Maloney et al., 1988; Varela and Bernays, 1988), and we believe it likely that insect cuticular lipids will be found to be similarly important for their natural enemies. This work is the initial study for a detailed examination of the role of the surface chemicals of *M. sexta* in relation to its predators.

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CHEMICAL ECOLOGY OF THE LUNA MOTH Effects of Host Plant on Detoxification Enzyme Activity

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Abstract—The effects of food plant on larval performance and midgut detoxification enzymes were investigated in larvae of the luna moth, *Actias luna*. Neonate larvae were fed leaves of black cherry, cottonwood, quaking aspen, white willow, red oak, white oak, tulip tree, paper birch, black walnut, butternut, or shagbark hickory. First instar survival, larval duration, and pupal weights were monitored as indices of food quality. Midgut enzyme preparations from fifth instars were assayed for β -glucosidase, quinone reductase, polysubstrate monooxygenase, esterase, and glutathione transferase activities. Larval survival on seven of the 11 plant species, including several recorded host plants, was extremely poor. Larvae performed well, and quite similarly, on birch, walnut, butternut, and hickory. Activities of all enzyme systems except β -glucosidase were significantly influenced by larval host plant. Of the systems assayed, quinone reductase and glutathione transferase activities were especially high. Comparisons of these values with published values for other Lepidoptera support the hypothesis that these enzyme systems are involved in conferring tolerance to juglone and related quinones occurring in members of the plant family Juglandaceae. Results suggest that host plant utilization by luna is more specialized at the individual or population level than at the species level and that biochemical detoxification systems may play a role in such specialization.

Key Words—*Actias luna*, Lepidoptera, Saturniidae, detoxification enzymes, enzyme induction, glutathione transferase, Juglandaceae, juglone, nutritional ecology, plant–insect interactions, quinone reductase.

INTRODUCTION

The luna moth, *Actias luna* L. (Saturniidae), occurs from southern Canada to Texas and Florida (Baker, 1972). As a species, it is moderately polyphagous, feeding on trees from at least eight plant families (Baker, 1972; Tietz, 1972). Little is known, however, of the relative suitability of various tree species as larval food plants. The research reported here was conducted in part to assess the influence of known or potential host plants on performance (survival and growth) of luna larvae.

A second objective of this research was to investigate host plant alteration of allelochemical-metabolizing enzymes in luna larvae. Detoxification of plant allelochemicals by specialized enzyme systems is widely believed to be one of the most important forms of insect adaptation to plant diets. Yet few studies have documented the enzymatic detoxification capacities of tree-feeding insects, and fewer still have addressed potential host plant effects on multiple enzyme systems.

These studies provided the opportunity to test several predictions about evolutionary and ecological adaptations of enzymatic detoxification systems to particular food plants. Prior to the onset of this study, I had observed that luna larvae perform well on black walnut (*Juglans nigra*) and shagbark hickory (*Carya ovata*). The most distinguishing secondary metabolites of these and other members of the Juglandaceae are juglone and related 1,4-naphthoquinones, compounds that are generally deterrent or toxic to insects (Gilbert et al., 1967; Hedin et al., 1980; Norris, 1986). The enzyme systems most likely responsible for detoxification of juglone in Juglandaceae-adapted insects include quinone reductase, which catalyzes the reduction of quinones to hydroquinones (Yu, 1987a), and glutathione transferase, which conjugates allelochemicals containing α,β -unsaturated carbonyls (e.g., juglone) with reduced glutathione (Wadleigh and Yu, 1987). These enzyme systems can be induced by dietary exposure to various allelochemicals. Thus I predicted that quinone reductase and glutathione transferase activities would (1) be especially high in luna larvae in comparison to published values for other polyphagous Lepidoptera, and (2) be induced in larvae fed members of the Juglandaceae, in comparison to larvae fed members of other plant families.

METHODS AND MATERIALS

Insects. *Actias luna* eggs were obtained from a gravid female moth captured in Laurel County, Kentucky, in May 1987. Larvae were reared in plastic boxes at ambient temperature on leaves of black walnut or shagbark hickory. Newly eclosed adult moths were paired to provide larvae for this study. To

introduce more genetic variability into the laboratory culture, an attempt was made to pair laboratory-reared females with local males by placing tethered females in nearby woodlots; these attempts proved unsuccessful.

Feeding Trials. Neonate larvae were placed onto fresh leaves of 11 species of trees, including six genera on which luna are known to feed, and two genera for which no previous feeding records exist (Table 1). Each replicate consisted of 10–14 larvae, with leaves, in a ventilated plastic Petri dish (15 × 2.5 cm); leaf petioles were inserted into florist's Water Piks to maintain leaf turgor. Leaves were replaced at two- to three-day intervals. When larvae reached the fourth stadium, they were transferred to larger shoebox cages. Larvae were reared in a Percival environmental chamber at 24°C, on a 12:12 light-dark cycle. Measurements were made of first instar survival, duration from hatching to spin-up of the cocoon, and pupal weight (six to seven days following spin-up) as indices of food plant quality.

Enzyme Preparation. Because larval midguts are the major site for detoxification of plant allelochemicals, they were used as the enzyme source. Midguts (5–9 per enzyme replicate) were dissected from fifth instar larvae (3 to 7 days old) and gut contents removed. Midguts were then washed (0.2 M phosphate buffer, pH 7.8) and homogenized by 10 strokes in a Ten Broeck tissue grinder. The homogenate was centrifuged at 10,000g (10 min) and the supernatant removed and centrifuged at 100,000g (60 min), to separate soluble (cytosolic) and microsomal (membrane-bound) enzymes. The enzyme preparations

TABLE 1. TREE SPECIES ASSAYED FOR LARVAL PERFORMANCE

Species assayed	Plant family	Luna previously recorded to feed on the: ^a		
		Species	Genus	Family
Black cherry (<i>Prunus serotina</i>)	Rosaceae		X	X
Cottonwood (<i>Populus deltoides</i>)	Salicaceae			X
Quaking aspen (<i>Populus tremuloides</i>)	Salicaceae			X
White willow (<i>Salix alba</i>)	Salicaceae		X	X
Red oak (<i>Quercus rubra</i>)	Fagaceae	?	X	X
White oak (<i>Quercus alba</i>)	Fagaceae	?	X	X
Tulip tree (<i>Liriodendron tulipifera</i>)	Magnoliaceae			
Paper birch (<i>Betula papyrifera</i>)	Betulaceae	X	X	X
Black walnut (<i>Juglans nigra</i>)	Juglandaceae	X	X	X
Butternut (<i>Juglans cinerea</i>)	Juglandaceae	X	X	X
Shagbark hickory (<i>Carya ovata</i>)	Juglandaceae	X	X	X

^aHost plant records compiled from Holland (1968), Baker (1972), and Tietz (1972). X = documented feeding; ? = probable feeding, not documented in preceding references.

were then processed according to Brattsten (1987a) for flash-freezing in liquid nitrogen and storage at -70°C . All procedures were conducted at $0-4^{\circ}\text{C}$.

Enzyme Assays. Assays were conducted for five different enzyme systems. β -Glucosidase activity was measured because these enzymes are involved in the metabolism of glycosidic allelochemicals (often activating rather than detoxifying them), and because little is known about host plant alteration of β -glucosidases (Lindroth, 1988). Quinone reductase (QR) and glutathione transferase (GT) activities were determined because they are likely to be especially important in the adaptation of luna larvae to members of the Juglandaceae. Polysubstrate monooxygenase (PSMO) and esterase activities were measured because these enzyme systems are generally important detoxification mechanisms in insects. Enzyme assays were optimized with respect to substrate and enzyme concentrations, incubation time, and buffer pH. Each enzyme assay for each enzyme solution was conducted in duplicate or triplicate. Specific activities were calculated relative to protein concentrations of the enzyme preparations, as determined by the Folin-phenol procedure of Schacterle and Pollack (1973).

β -Glucosidase activity was measured according to Lindroth (1988). Each 1 ml incubation solution contained 50–175 μg protein, 50 μmol salicin, and 0.1 M potassium phosphate buffer (pH 6.0). Solutions were incubated for 30 min at 35°C . Glucose liberated from salicin by β -glucosidase activity was quantified enzymatically (Sigma Diagnostic Kit 315).

Quinone reductase (QR) activity of soluble and microsomal fractions was measured using the juglone-dependent NADPH oxidation method of Yu (1987a). First, 40–70 μg protein, 160 μl NADPH (2.5 mM in phosphate buffer), and 0.1 M sodium phosphate buffer (pH 8.0) were mixed together for a total volume of 2 ml. From that solution, 995 μl were placed into a sample cuvette and mixed with 5 μl juglone (10 mM in methyl cellosolve). The remaining solution was placed into the reference cuvette. NADPH oxidation was determined as the decrease in absorbance at 340 nm over several minutes; a value of 6.22/mM/cm was used for the extinction coefficient of NADPH (Segel, 1976). Use of a double-beam spectrophotometer canceled out effects of endogenous NADPH oxidation because both cuvettes contained enzyme and NADPH. To confirm that the observed activity was due to a reductase rather than a microsomal oxidase, QR activities were compared between an untreated microsomal enzyme solution and a solution that had been bubbled with carbon monoxide (2 bubbles/min for 1 min). No difference was found in NADPH oxidation between the two samples.

Cytochrome *c* reductase and *O*-demethylase activities of microsomal fractions were measured as indices of PSMO activity. Cytochrome *c* reductase activity was quantified as the production of reduced cytochrome *c* with time, according to Brattsten et al. (1980). Assay solutions (1 ml) consisted of 20–50 μg protein, 200 μl cytochrome *c* (4 mg/ml in phosphate buffer), 100 μl NADPH

(1 mM in phosphate buffer), and 0.1 M potassium phosphate buffer (pH 8.0). Production of reduced cytochrome *c* was measured as the increase in absorbance (550 nm) over 1 min versus a blank containing everything but the enzyme. No significant endogenous reduction of cytochrome *c* was observed with this procedure. A value of 27.6/mM/cm was used for the extinction coefficient of reduced cytochrome *c* (Margoliash and Frohwirt, 1959). The assay for *O*-demethylase activity was adapted from Hansen and Hodgson (1971) and measured the PSMO-catalyzed formation of *p*-nitrophenol from *p*-nitroanisole. Each assay solution contained 0.5–1.0 mg protein; an NADPH-generating system consisting of 0.36 mM NADP, 3.6 mM glucose-6-phosphate, and 2 units/ml glucose-6-phosphate dehydrogenase (final assay concentrations); 0.15 M potassium phosphate buffer (pH 7.8, with 1 mM EDTA) to a total volume of 780 μ l; and 20 μ l *p*-nitroanisole (32 mM in methyl cellosolve) to initiate the reaction. Enzyme solutions were incubated at 32°C for 30 min; reactions were terminated with 200 μ l 1 M HCl. Acidified solutions were extracted with 1 ml dichloromethane, and these in turn were extracted with 900 μ l 0.5 M NaOH. Absorbance was read at 400 nm and converted to concentration of *p*-nitrophenol using a standard curve.

Soluble and microsomal esterase activities were quantified as described by Brattsten (1987a). Assay solutions contained 0.2–1.0 μ g protein and 0.05 M sodium phosphate buffer (pH 8.0) to a total volume of 495 μ l. Assays were initiated with addition of 5 μ l 1-naphthyl acetate (50 mM in ethanol). Reactions were run at 32°C for 10 min and terminated with addition of a 1 ml solution of fast blue B and sodium dodecyl sulfate (0.2 and 7.5 mg/ml water, respectively). After 10 min, absorbance was read at 600 nm and converted to concentration of 1-naphthol using a standard curve.

Soluble and microsomal glutathione transferase activities were measured as the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione, as documented previously (Lindroth, 1989). Reaction mixtures consisted of 4–15 μ g protein, 50 μ l glutathione (0.05 M in phosphate buffer), and 0.1 M potassium phosphate buffer to a total volume of 975 μ l. Reactions were initiated with addition of 25 μ l CDNB (40 mM in methyl cellosolve) and the increase in absorbance (340 nm) monitored for 1 min against a blank containing everything but enzyme. Again, use of a double-beam spectrophotometer automatically accounted for changes in absorbance due to nonenzymatic conjugation of CDNB. Concentrations of CDNB-glutathione were calculated using an extinction coefficient of 9.6/mM/cm (Cohen, 1986).

Statistics. Statistical analyses were performed with the SYSTAT statistical software package. One-way analyses of variance and multiple comparisons among means were conducted using a multiple general linear model (Wilkinson, 1987). For results from the survival trials, percentages were transformed ($\arcsin \sqrt{y}$) prior to analysis. For all other experiments in which Bartlett's test

of homogeneity indicated unequal distribution of variance, data were transformed ($\ln y$) prior to analysis.

RESULTS

Feeding Trials. Performance of luna larvae was exceptionally poor on seven of the 11 plant species tested (Table 2). For six of these species, no larvae survived through the first stadium. Survival of larvae fed willow was 33% for one replicate, but 0% for all others. Larvae fed quaking aspen or red oak consumed small amounts of leaf material prior to death, whereas larvae fed black cherry, cottonwood, or tulip tree showed few, if any, signs of feeding. In contrast, larvae fed birch or any of the three members of the Juglandaceae exhibited very high first instar survival.

Among the larvae that survived to pupation, those fed butternut had the shortest larval duration, but also the lowest average pupal weight (Table 2). Larvae fed birch required the longest time prior to pupation and had intermediate pupal weights. Luna reared on black walnut had the highest pupal weights (43% higher than those reared on the congeneric butternut) and intermediate larval development times.

Enzyme Assays. Survival of larvae was sufficiently high in only four of the 11 treatments to provide enough fifth instars for enzyme assays. Larval food plant did not affect activity of midgut β -glucosidases (Table 3), but it did influence activity of all the detoxification enzyme systems.

TABLE 2. PERFORMANCE OF *Actias luna* LARVAE ON VARIOUS FOOD PLANTS^a

Species	First instar survival (%)	Larval duration (days)	Pupal weight (g)
Black cherry	0.0 (5)		
Cottonwood	0.0 (5)		
Quaking aspen	0.0 (5)		
White willow	5.6 \pm 5.6(5)a	31.3 (1)	2.20 (1)
Red oak	0.0 (5)		
White oak	0.0 (5)		
Tulip tree	0.0 (5)		
Paper birch	98.1 \pm 1.3(9)b	31.9 \pm 0.8(7)c	2.25 \pm 0.12(7)a
Black walnut	96.3 \pm 1.5(9)b	28.8 \pm 0.5(8)b	2.85 \pm 0.15(8)b
Butternut	92.1 \pm 3.5(5)b	26.3 \pm 0.6(4)a	2.00 \pm 0.19(4)a
Shagbark hickory	97.6 \pm 1.5(7)b	29.7 \pm 0.4(7)b	2.49 \pm 0.17(7)a,b

^aValues represent $\bar{X} \pm 1$ SE; sample sizes shown in parentheses. Within a column, means followed by different letters are significantly different ($P < 0.05$).

TABLE 3. SPECIFIC ACTIVITIES ($\bar{X} \pm 1$ SE) OF MIDGUT ENZYMES IN *Actias luna* LARVAE FED VARIOUS HOST PLANTS^a

Host plant	β -Glucosidase		Quinone reductase		Cytochrome c reductase	O-Demethylase		Esterase		Gluthathione transferase	
	Soluble	Microsomal	Soluble	Microsomal		Soluble	Microsomal	Soluble	Microsomal	Soluble	Microsomal
Paper birch (5)	98.6a ± 11.6	288.3b ± 17.3	47.0a ± 2.0	288.3b ± 17.3	169.9b ± 7.8	249.6c ± 30.0	1072a ± 48	1192.6c ± 86.7	1016a ± 86	160.4a ± 16.4	
Black walnut (5)	126.2a ± 10.7	195.9a ± 7.9	61.3c ± 2.7	195.9a ± 7.9	124.1a ± 4.2	179.9b ± 24.2	1940d ± 111	665.6a ± 30.8	2246b ± 82	258.3b,c ± 18.2	
Butternut (4)	108.4a ± 15.4	173.9a ± 9.6	53.1b ± 1.2	173.9a ± 9.6	139.1a ± 15.3	94.4a ± 16.6	1337b ± 28	691.3a,b ± 26.1	2867b ± 378	226.1b ± 13.0	
Shagbark hickory (5)	118.9a ± 9.3	255.0b ± 8.7	55.7b ± 0.9	255.0b ± 8.7	147.7a,b ± 10.0	145.2a,b ± 12.4	1619c ± 89	854.6b ± 37.3	2491b ± 86	282.4c ± 21.4	

^aSample sizes shown in parentheses. All specific activities except O-demethylase shown as nmol/min/mg protein. O-Demethylase activities shown as pmol/min/mg protein. Within a column, means followed by different letters are significantly different ($P \leq 0.05$).

Both soluble and microsomal quinone reductase activities were affected by larval host plant (Table 3). Soluble QR activity was lowest in larvae fed birch and highest in those fed walnut, a 1.3-fold difference. Microsomal QR activity, however, was lowest in larvae fed butternut and highest in those fed birch, a 1.7-fold difference. Specific activities (per unit protein) were three to six times higher in microsomal fractions than in soluble fractions. However, given that total soluble protein averaged six to eight times that of microsomal protein, total soluble QR activity was higher than total microsomal QR activity in larvae from all treatments.

Cytochrome *c* reductase and *O*-demethylase assays showed that highest PSMO activities occurred in larvae reared on birch (Table 3). Lowest cytochrome *c* reductase levels were in larvae fed walnut, whereas lowest *O*-demethylase activities were in larvae fed butternut. Host plant effects on enzyme activity accounted for a 1.4- and 2.6-fold variation in cytochrome *c* reductase and *O*-demethylase activities, respectively.

Activities of soluble esterases were significantly different among all treatments and were 1.8-fold higher in larvae fed walnut than in larvae fed birch (Table 3). Microsomal esterases exhibited an opposite trend in activity, with lowest values in larvae fed walnut, and highest in those fed birch. Specific activities of soluble esterases were higher than those of microsomal esterases in all treatments except birch. Considering the much larger quantity of soluble protein than microsomal protein in the enzyme preparations, a preponderance of midgut esterase activity is effected by soluble enzymes.

Glutathione transferase (GT) activities were strongly correlated with host plant genus (Table 3). There were no significant differences in soluble GT activities among larvae fed walnut, butternut, or hickory. Activities exhibited by these larvae were 2.2- to 2.8-fold higher than those of larvae fed birch. Similarly, variations in activities of microsomal GTs among larvae fed members of the Juglandaceae were small in comparison to the difference between those larvae and larvae reared on birch.

With respect to major among-treatment trends in enzyme activities, few patterns are evident among larvae fed species of the Juglandaceae. Clearly though, the enzyme profile of larvae reared on birch differed substantially from those of larvae reared on walnut, butternut, or hickory. Birch-fed larvae showed the lowest β -glucosidase, soluble QR, and soluble and microsomal esterase and GT activities, but the highest cytochrome *c* reductase, *O*-demethylase, and microsomal QR activities.

DISCUSSION

Larval performance on the trees tested in this study differed significantly from that expected on the basis of published food plant records (Holland, 1968; Baker, 1972; Tietz 1972). Survivorship values of 0% on white oak and red oak,

and of 5.6% on willow, were particularly surprising. Larvae performed quite well, and fairly similarly, on birch and the three species of Juglandaceae. These divergent results are best explained by the low genetic variability in the laboratory population. Although *Actias luna* feeds on a variety of plant families throughout its geographic range, local populations or individuals may specialize on particular plant families. Scriber and Feeny (1979) suggested that a similar situation exists for other species of saturniids. This study lends additional support to the notion of Fox and Morrow (1981) that insects exhibiting generalized diets at the species level are likely to have specialized diets at the population level.

Little is known about host plant alteration of midgut enzymes in deciduous tree-feeding insects. These data show that, with one exception, larval food plant strongly affects the activity of midgut enzymes. The single exception was β -glucosidase activity, which was not significantly affected by the plant species consumed. This lack of response contrasts with results from a similar study (Lindroth, 1988) in which strong host plant effects on β -glucosidase activity were found in the eastern tiger swallowtail (*Papilio glaucus glaucus*).

Although larval food plant significantly affected quinone reductase activities, the effects were not strong (1.3- and 1.7-fold maximum differences for soluble and microsomal QR activities, respectively). Similarly, Yu (1987a) found that individual allelochemicals induced QR activities in fall armyworm 1.3- to 2.5-fold. Quinone reductase activities in luna larvae were markedly higher than those found by Yu (1987a) in other lepidopteran species. Soluble QR activities in luna were up to 2.3-fold higher than those in the armyworm, and microsomal activities in luna were 5.6–6.2 times higher than those in four other species.

Plant alteration of PSMO-related activities was low to moderate in the luna larvae. Cytochrome *c* reductase activities and induced responses were similar to those reported for *Papilio glaucus glaucus* fed various host plants (Lindroth, 1989). *O*-Demethylase activities were also in the range reported for *P. g. glaucus* (Lindroth, 1989), for fall armyworm fed various crop plants (Yu, 1983), and for fall armyworm, corn earworm, tobacco budworm, and velvetbean caterpillar reared on artificial diets (Yu, 1987b).

Published studies of host plant alteration of insect esterase activities have typically shown small to moderate effects (Yu, 1986, 1987b; Brattsten, 1987b). In the present study maximum differences of 1.8-fold were found for both soluble and microsomal esterases. The range of esterase activities in luna larvae was similar to that of *P. g. glaucus* fed various host plants (Lindroth, 1989) and to the single value of 1004 nmol/min/mg protein reported for gypsy moth (*Lymantria dispar*) reared on an artificial diet (Kapin and Ahmad, 1980).

Soluble glutathione transferases exhibited the strongest apparent induction response of the enzymes assayed in this study, although the possibility exists that GT activity was simply inhibited in birch-fed larvae. In addition, the overall soluble GT activity of larvae fed members of the Juglandaceae was quite

high in comparison to that of other insect species. Midgut glutathione transferase activities (measured by the CDNB assay) for *P. g. glaucus*, the southern armyworm, fall armyworm, and corn earworm ranged from 600 to 970 nmol/min/mg protein (Gunderson et al., 1986; Brattsten, 1987a; Yu, 1987b; Lindroth, 1989).

Although this was a preliminary study, the results suggest that evolutionary and ecological adaptations of luna to particular food plants involve alterations in biochemical detoxification systems. Moreover, the enzyme systems of particular significance here have rarely demonstrated such importance in other plant-insect associations. Unusually high constitutive levels of quinone reductase and glutathione transferase activities in luna are probably an evolutionary adaptation to the presence of juglone and related quinones in their preferred food plants (Juglandaceae). The fact that soluble QR and soluble and microsomal GT activities appeared to be induced in larvae fed members of the Juglandaceae, relative to those fed birch, shows that biochemical alterations are involved in the adaptations of individual larvae to specific foods. These types of biochemical adjustments may well be involved in local feeding specialization of regionally generalist insects. Finally, this study illustrates that an understanding of the dominant allelochemicals in a plant-insect system can give clues as to the type of biochemical adaptations exhibited by the insects.

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ISOLATION AND IDENTIFICATION OF A COMPOUND FROM SOYBEAN CYST NEMATODE, *Heterodera glycines*, WITH SEX PHEROMONE ACTIVITY

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Abstract—A single compound with sex pheromone activity was isolated from the female soybean cyst nematode, *Heterodera glycines*, by a sequence of four high-performance liquid chromatographic steps and identified as vanillic acid by a combination of ultraviolet spectroscopy and chromatography. The structure was confirmed by gas chromatography-mass spectrometry. Both attractancy and coiling behavior in male soybean cyst nematode were elicited by authentic vanillic acid.

Key Words—Soybean cyst nematode, *Heterodera glycines*, Nematoda, sex pheromone, vanillic acid, attractancy, coiling, bioregulation, behavior.

INTRODUCTION

The identification of behavior-modifying compounds of free-living, animal- and plant-parasitic nematodes has been the subject of intensive study since the late 1960s. However, to date, no complete structures of any such compounds have been reported (for review see Bone, 1987; Huettel, 1986). Previous studies on nematodes generally have centered on sex pheromones, aggregation pheromones, or epideitic responses (Huettel, 1986).

Bone et al. (1979), in extensive studies of the animal parasitic nematode, *Nippostrongylus brasiliensis*, tentatively identified a long-chain polypeptide as the sex attractant of this nematode. In studies of the free-living nematode, *Caenorhabditis elegans*, compounds with physical and chromatographic properties

similar to hydroxylated, short-chain fatty acids and bile acids were partially characterized as a primer or epideitic pheromone that causes the dauer larval formation in this species (Golden and Riddle, 1982, 1984). Bone (1986) fractionated the sex pheromone of a plant-parasitic nematode of soybeans, *Heterodera glycines*, into two components of different solubility.

Our laboratory also has investigated pheromone production in *H. glycines*, the soybean cyst nematode (SCN). We have developed more sensitive bioassays than those used previously to detect responses by males to females of this species (Huettel and Rebois, 1986; Huettel and Jaffe, 1987). Unlike previous bioassays (Greet et al., 1968; Rende et al., 1982), which required at least 8 hr or more to complete, we developed a rapid bioassay based on a specific behavior that could be completed in several minutes. Furthermore, we demonstrated that this behavior could be induced in males only by the presence of females or their crude extracts (Huettel and Jaffe, 1987). This rapid method allowed us to screen large numbers of high-performance liquid chromatography (HPLC) fractions of female extracts.

We report here the isolation and complete structural identification of a compound (SCNP) from female soybean cyst nematode with sex pheromone activity. This is, to our knowledge, the first structure of this type to be reported from a plant-parasitic nematode.

METHODS AND MATERIALS

Soybean Cyst Nematode Culture and SCNP Extraction. *H. glycines* Ichinohe (Nematoda), race 3, was maintained on monoxenic soybean root explant cultures, as previously described (Huettel and Rebois, 1985). Female nematodes (1000), ca. 10–12 days old, were removed manually from the roots and placed in 15 ml Milli-Q water (Millipore). The nematodes were gently agitated in the dark at room temperature for 24 hr. The aqueous extract was then filtered through a 0.22- μ m Acrodisc filter (Gelman) and frozen at -80°C in a 20-ml polypropylene vial. Batches of extract from 20,000–40,000 females were lyophilized at $0-5^{\circ}\text{C}$.

SCNP Purification. The lyophilisate from 23,870 females was taken up in two 3-ml portions of the starting gradient mixture of HPLC step A (see below). The resulting solution was filtered through a 0.45- μ m Millex-HV filter (Millipore) prior to analysis by HPLC.

HPLC Step A. The filtered sample (5.3 ml) was divided into five equal parts (4774 female equivalents) each of which was chromatographed on a 4.6 \times 250 mm, 5 μ m Supelcosil LC-18DB column with a Pelliguard guard column (Supelco) on a model 840 liquid chromatograph with autosampler (Waters). The sample was injected onto the column and eluted with a concave gradient (Waters

curve 7) from 10 to 60% acetonitrile (0.1% v/v trifluoroacetic acid) against 0.1% aqueous trifluoroacetic acid (TFA) over 1 hr at ambient temperature and 1.0 ml/min. The eluent was monitored spectrophotometrically at 214 nm. Fractions were collected over 1-min intervals with both the autosampler and fraction collector cooled to 0–5°C. Fractions with the same retention times from the five runs were pooled in the fraction collector, and those fractions (17–19 min) with biological activity were lyophilized at 0–5°C.

HPLC Step B. Each of the pooled lyophilized 1-min fractions was purified further by reverse-phase HPLC on a 4.6×250 -mm, 7- μ m Zorbax C-8 150 SP column (Dupont) on a 1090 liquid chromatograph equipped with a photodiode array detector and Chemstation (Hewlett-Packard). The column was eluted with a linear gradient from 2 to 15% acetonitrile in 0.25 N triethylammonium phosphate (TEAP), pH 2.20, over 1 hr at 28°C and 0.4 ml/min. Fractions were collected at 1-min intervals, and the fractions (49–50 min) containing SCNP were processed in vacuo in a model SVC200H Speed Vac concentrator (Savant) to remove acetonitrile.

HPLC Step C. Fractions containing partially purified SCNP were purified further by reverse-phase HPLC using the same 1090 chromatograph and Zorbax column with a linear gradient from 2 to 20% acetonitrile (0.1% v/v TFA) against 0.1% aqueous TFA over 1 hr at 28°C and 0.4 ml/min. Fractions were collected at 1-min intervals and the fraction (46–47 min) containing SCNP was evaporated to dryness in the Speed Vac.

HPLC Step D. Final purification of the SCNP was achieved by reverse-phase HPLC of the partially purified SCNP fractions resulting from HPLC step C using the same 1090 chromatograph and Zorbax column, but using a linear gradient from 2 to 20% 2-propanol (0.1% v/v TFA) against 0.1% aqueous TFA over 1 hr at 28°C and 0.4 ml/min. The resulting fractions containing the SCNP peak were evaporated to dryness in the Speed Vac and stored at –80°C.

Control HPLC Isolation Experiments. Three control samples were prepared and analyzed by HPLC as follows: (1) Milli-Q water (200 ml) was lyophilized, taken up in starting gradient mixture of HPLC step A, and analyzed by HPLC step A. Fractions eluting between 17 and 20 min were pooled, concentrated, and analyzed by HPLC step B. (2) Sterile *Glycine max* (L.) CV Kent root (1 g) was stirred in 15 ml Milli-Q water at room temperature in the dark for 24 hr. The aqueous extract was filtered through a 0.22- μ m Acrodisc filter and lyophilized. The residue was analyzed by HPLC step A. Fractions eluting between 16 and 20 min were pooled, concentrated, and analyzed by HPLC step B. (3) Gamborg's B-5 medium Agar (Gibco) was prepared according to the manufacturer's instructions. A 1-g sample was stirred in 15 ml Milli-Q water at room temperature in the dark for 24 hr. The aqueous phase was filtered and analyzed by HPLC step A. Fractions eluting between 16 and 20 min were concentrated and analyzed by HPLC step B.

HPLC Analysis of Substituted Phenolic Acids. A group of commercially available substituted phenolic acids (Aldrich) was analyzed by the conditions of HPLC step D. The following absorbance maxima and retention times were observed: 2,3-dihydroxybenzoic acid, 206, 244, 247, 317 nm and 43.8 min; 2,4-dihydroxybenzoic acid (β -resorcylic acid), 208, 256, 295 nm and 43.0 min; 2,5-dihydroxybenzoic acid (gentisic acid), 212, 238, 330 nm and 34.5 min; 2,6-dihydroxybenzoic acid (γ -resorcylic acid), 208, 215, 247, 308 nm and 40.5 min; 3,4-dihydroxybenzoic acid (protocatechuic acid), 206, 217, 260, 294 nm and 23.4 min; 3,5-dihydroxybenzoic acid (α -resorcylic acid), 204, 220, 248, 307 nm and 23.4 min; 3,4-dimethoxybenzoic acid (veratric acid), 204, 219, 260, 293 nm and 56.0 min; 3-hydroxy-4-methoxybenzoic acid (isovanillic acid), 205, 218, 261, 294 nm and 40.4 min; 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 205, 218, 261, 293 nm and 37.0 min.

Bioassay. Dried aliquots of HPLC fractions were taken up in Milli-Q water for bioassay by our previously described method (Huettel and Jaffe, 1987). The response of male SCN to various concentrations of vanillic acid (VA) was bioassayed as follows: male SCN were removed by hand from soybean root culture and aerated in 200 ml Milli-Q water to remove any effects of previous exposure to pheromone. For each concentration of VA in Milli-Q water, 10 males were removed from the water after aeration and placed in a 20- μ l drop of VA solution on a 1.5% Noble Agar plate (Difco). After 30 sec, the males were removed from the VA solution and observed for coiling behavior on the agar plate with a stereomicroscope (Nikon).

Derivatization Procedure for GC-MS Analysis of SCNP. A dried aliquot of the purified SCNP peak eluting at 37.0 min in HPLC step D (see Figure 4) was transferred by means of MeOH washes (2 \times 0.1 ml) to a 10-cm \times 1.6-cm-OD, Teflon-lined, screw-capped, glass culture tube for derivatization by a procedure adapted from the methods of Woolson and Harris (1967) and Metcalfe and Schmitz (1961). After the addition of 0.45 g BF₃-MeOH reagent (Supelco), the tube was tightly capped and heated in a boiling-water bath for 3 min. After cooling, 6 ml of 10% Na₂SO₄ was added to decompose excess reagent. The aqueous phase was extracted with 3 ml iso-octane-benzene (2:1). The organic extract was centrifuged for 5 min at 3500 rpm and the resulting supernatant transferred to a 15-ml conical flask for removal of solvents by rotary evaporation at 25°C. The residue was transferred by means of two EtOAc washes (0.15 and 0.10 ml) to a 0.3-ml glass, tapered-tip, HPLC autoinjector vial (Waters). After removal of solvent on the Speed Vac, the residue was dissolved in 30 μ l EtOAc and 2 μ l of the resulting solution was analyzed by GC-MS.

The following control samples were derivatized and analyzed by GC-MS: (1) dried fractions eluting before (35–36 min) and after (39–40 min) the SCNP peak, (2) authentic VA (200 mg), and (3) a solvent/reagent blank.

TABLE 1. GC-MS-SIM ANALYSIS OF METHYL ESTER DERIVATIVES OF SCNP ISOLATED FROM *Heterodera glycines* AND VANILLIC ACID

Compound	Average retention time of GC peak (min) ^a	Relative Abundance (%) at Indicated Ion			
		<i>m/z</i> 108.1	<i>m/z</i> 123.2	<i>m/z</i> 151.3	<i>m/z</i> 182.2
SCNP	5.00	13.93	27.27	98.57	96.77
Vanillic acid (VA)	4.99	14.07	28.27	100.0	94.17
Methyl vanillate (MEVA) ^b	5.01	13.41	26.69	99.82	94.86

^aFor SCNP, *N* = 3; VA, *N* = 3; MEVA, *N* = 11.

^bAuthentic sample (underivatized).

GC-MS Analysis. Samples were analyzed by GC-MS on a model 5985A GC-MS equipped with a model 18835 capillary inlet system, model 1000 computer, model 7920 and 7906 disk drives (Hewlett-Packard), and a DB5 30-m × 0.257-mm-ID fused-silica capillary (0.25 μm film) column (J&W), operated in the splitless mode and temperature programmed from 70 to 250°C at 18°C/min. For selected ion monitoring (SIM) experiments, the column was temperature programmed from 70 to 250°C at 30°C/min. The column was held at 250°C for 20 min between runs for column burn-off. GC-MS instrument parameters were: injection port, 250°C; carrier gas (He) inlet pressure, 8 psi; detector source temperature, 200°C; electron multiplier voltage 2800 V. Major ions (relative intensities >4.0) for methyl vallinate (MEVA) standard were observed at *m/z* 108 (12.6), 111 (5.3), 123 (23.5), 136 (4.8), 151 (base, 100.0), 152 (10.8), 167 (4.5), 182 (M+, 55.9), and 183 (4.4). For SIM analyses, the instrument was calibrated to monitor exact masses of the following ions: *m/z* 108.1, 123.2, 151.3, and 182.2. A total of 200-msec dwell time (50 msec/ion) was used for each SIM scan. The GC retention times and relative abundances of the selected ions (Table 1) were averaged for the SCNP (*N* = 3), VA (*N* = 3), and MEVA (*N* = 11).

RESULTS AND DISCUSSION

A single compound (SCNP) with sex pheromone activity in male SCN was isolated from aqueous extracts of females and purified by a four-step reverse-phase HPLC procedure. Biologically active 1-min fractions eluting between 17 and 19 min by HPLC step A (Figure 1) were each further purified by HPLC step B. As shown (Figure 2) for the fraction eluting between 18 and 19 min, the change of column and buffer from TFA to TEAP at this stage of purification

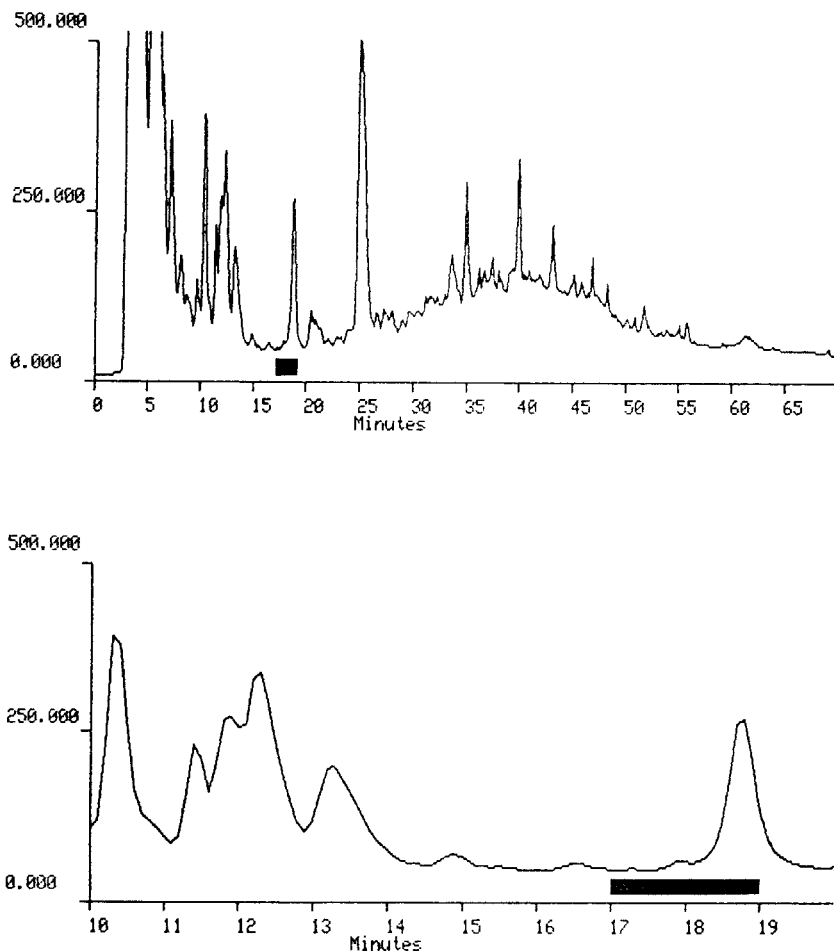


FIG. 1. Elution profile of extract of 4774 female soybean cyst nematodes by HPLC step A ($0.500A_{214}$ full scale) between 0 and 70 min (upper chromatogram) and 10–20 min (lower chromatogram). Bars indicate fractions with biological activity.

caused the fraction to resolve into several major peaks. The peak eluting between 49 and 50 min (later shown to be biologically active) was purified further by HPLC step C (Figure 3), where it eluted between 46 and 47 min. Although the change of buffer from TEAP to TFA at this stage of purification caused a co-eluting small peak in HPLC step B to shift to a lower retention time, satisfactory purification of the main peak was not achieved, as indicated by photodiode array UV spectroscopy. Final peak purification, however, was achieved in HPLC step D using the volatile TFA–2-propanol buffer (Figure 4). Superimposition

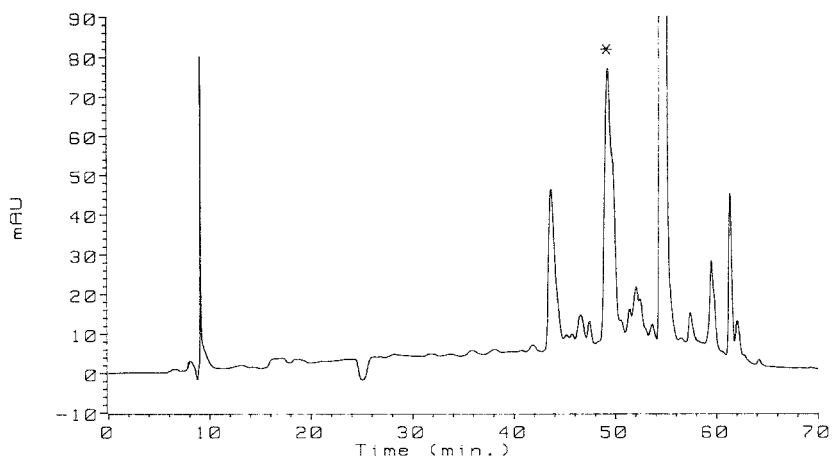


FIG. 2. Elution profile using HPLC step B ($0.100A_{220}$ full scale) of pooled fractions from HPLC step A eluting between 18 and 19 min from 23,870 females. Asterisk indicates peak with biological activity.

of the normalized upslope, apex, and downslope UV spectra (Figure 5) indicated peak homogeneity. Biological activity corresponded exactly with this peak.

The structure of SCNP was determined by a combination of UV spectroscopy and HPLC and confirmed by GC-MS. The UV spectrum of SCNP between

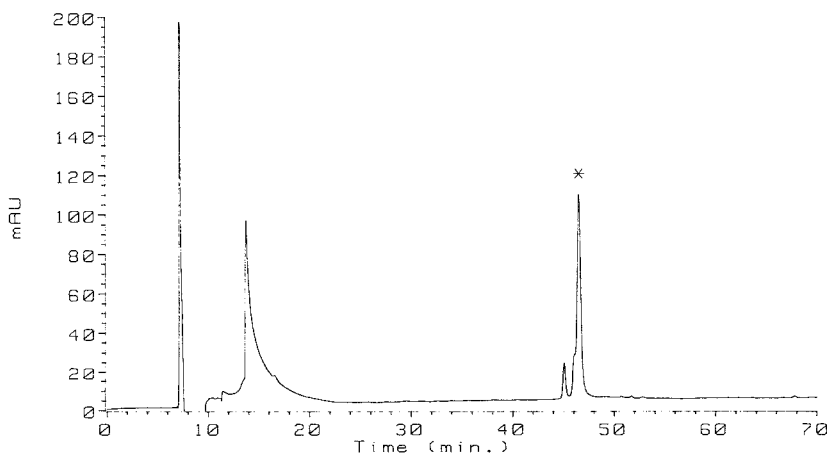


FIG. 3. Elution profile using HPLC step C ($0.200A_{220}$ full scale) of peak from HPLC step B eluting between 49 and 50 min. Asterisk indicates peak with biological activity.

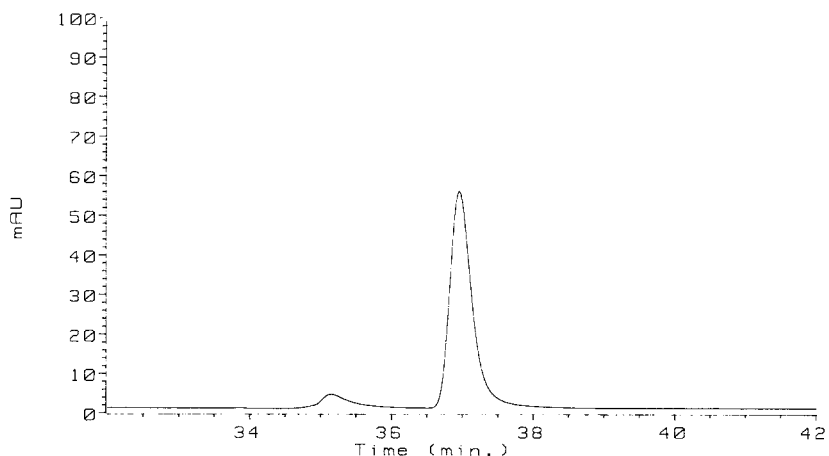


FIG. 4. Elution profile using HPLC step D ($0.100A_{260}$ full scale) between 32 and 42 min of purified SCNP from 23,870 females.

200 and 500 nm displayed four distinct maxima, characteristic of substituted phenolic acids (Doub and Vandenberg, 1955; Scott, 1961). Analysis of a representative group of these compounds under HPLC conditions of step D (see Methods and Materials) revealed that VA and SCNP had identical HPLC retention times and UV spectra. In addition, in higher pH buffers, such as 0.01 M NH_4OAc , at pH 6.55, both SCNP and VA exhibited identical changes in UV

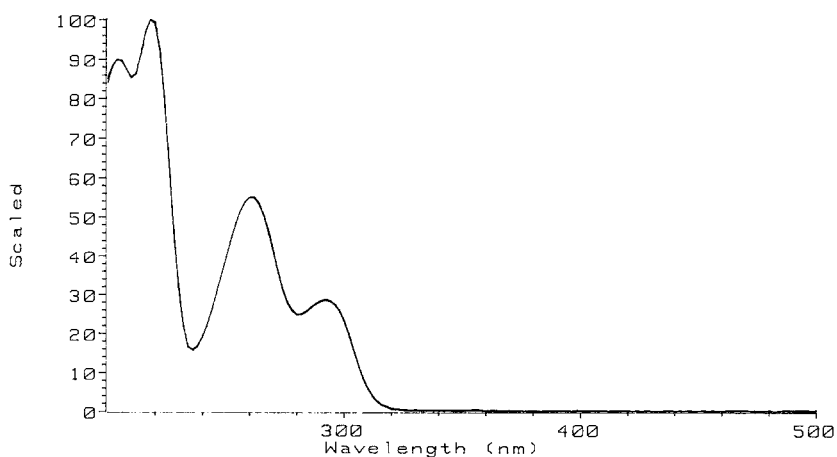
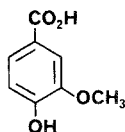


FIG. 5. Overlay of normalized UV spectra of the upslope, apex, and downslope of purified SCNP peak shown in Figure 4.

spectrum and shifts to lower HPLC retention times characteristic of substituted phenolic acids (data not shown). Based on these data, SCNP was identified as VA (4-hydroxy-3-methoxybenzoic acid) (Scheme 1).



SCHEME 1.

The total isolated yield of VA from 23,870 female SCN was 315 ng or 3.2 pg/female. In further support of the structural identification, the elution profile of a sample containing equal amounts of SCNP and VA (analyzed by the HPLC conditions of step C or D) displayed only one peak (Figure 6). In each case, superimposition of the normalized upslope, apex, and downslope UV spectra was observed, indicating peak homogeneity.

The identity of SCNP was confirmed by GC-MS. Because VA exhibited poor chromatographic behavior (broad, distorted peaks) on a variety of fused-silica capillary columns adaptable to GC-MS, it was converted by treatment with $\text{BF}_3\text{-MeOH}$ to its methyl ester (MEVA). As expected, MEVA eluted as a sharp symmetrical peak and was easily detectable by GC-MS in the low nanogram range (1–5 ng) when analyzed in the selected ion monitoring (SIM) mode.

Data in Table 1 show that $\text{BF}_3\text{-MeOH}$ reaction with the SCNP or an

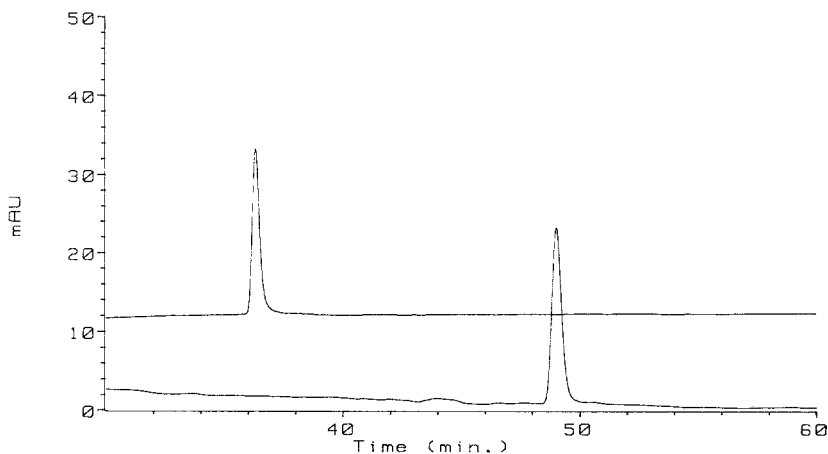


FIG. 6. Elution profiles between 30 and 60 min of equal amounts of purified SCNP and vanillic acid by conditions of HPLC step C ($0.050A_{220}$ full scale, lower chromatogram) and HPLC step D ($0.050A_{260}$ full scale, upper chromatogram).

authentic sample of VA produced a GC peak with essentially identical retention time and SIM profile as that observed for MEVA. In addition, dried fractions eluting before and after the SCNP peak in HPLC step D were also derivatized and analyzed by GC-MS-SIM. MEVA was not detected in either of these control fractions or the reagent/solvent blank.

Since SIM experiments consumed a small portion of the derivatized SCNP sample, the remainder was concentrated and analyzed by GC-MS in the full spectrum mode. Peak I (Figure 7) in the total ion chromatogram was identified as MEVA based on a comparison of its retention time and mass spectrum to those of an authentic sample of MEVA. All major ions present in MEVA (mass spectrum B) were observed in the mass spectrum of peak I (mass spectrum A) in approximately the same relative intensity.

Because the yield in the BF_3 -MeOH derivatization reaction was 55%, we expected the presence of a significant amount of underivatized SCNP in the reaction mixture. Examination of spectra in the region (peak II, 5.85 min) in the total ion chromatogram where VA elutes as a broad, unsymmetrical peak (5.18–5.92 min) provided a spectrum (mass spectrum C) that contained all major ions observed for an authentic sample of VA (mass spectrum D), including the molecular ion m/z 168 and the base peak at m/z 153 corresponding to $\text{M} - \text{CH}_3$. Clearly, the GC-MS and GC-MS-SIM data confirm VA as the SCNP. Peaks other than I and II in the total ion chromatogram (Figure 7) resulted from HPLC column bleed and/or impurities in the reagents/solvents.

Male SCN displayed identical sex pheromone behavior to VA, i.e., the previously observed attractancy and coiling (Huettel and Rebois, 1986; Huettel and Jaffe, 1987) towards females or their extracts. Males responded to a concentration range of 10^{-5} – 10^{-7} M VA. At concentrations $>10^{-5}$ M VA, a paralytic-like effect was observed, resulting possibly from an "overload" of the pheromone receptor(s). It should be noted that VA is extremely active in our bioassay, with 60% of the males tested responding to 10^{-7} M VA or a total of 336 pg.

The phenolic acids including VA are ubiquitous compounds in nature, occurring in soil (Flaig et al., 1975) as products of plant lignin decomposition by fungi (Ishihara and Miyazaki, 1972; Ander and Eriksson, 1978). Although previous work (Harden and Stutte, 1980; Porter, 1983) had failed to detect VA in extracts of unhydrolyzed soybean leaf and root, it was considered essential nevertheless to conduct control experiments. These included the elimination of soybean root, Milli-Q water, and agar as a source of VA by our purification procedure. Both the weight of root and agar used in the control experiments were estimated to be substantially greater than the total weight of the females used in the SCNP isolation. The volume of Milli-Q water was about half that used in the SCNP isolation. Results of these control experiments were negative, confirming only the female SCN as the source of VA.

Although a recent report (Bone, 1986) suggested a two-component sex

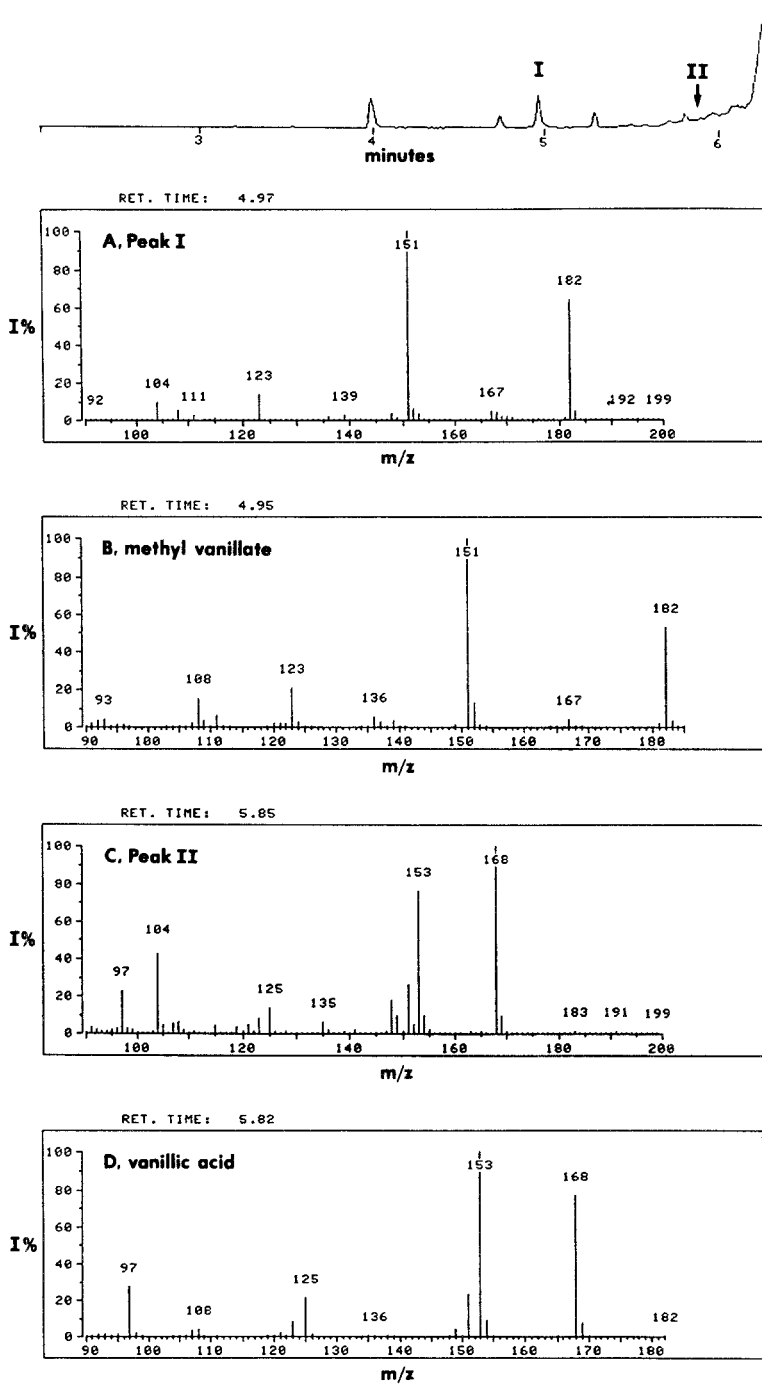


Fig. 7. Total ion chromatogram of derivatized SCNP (upper trace) and associated mass spectra: (A) peak I; (B) methyl vanillate; (C) peak II; (D) vanillic acid.

pheromone for SCN, our isolation procedure yielded only a single compound that elicited both aspects of SCN sex pheromone behavior, i.e., attractancy and coiling. We do not, however, rule out the possibility of other active compounds undetected by us.

To our knowledge, vanillic acid has not been previously identified as having sex pheromone activity in any species, although vanillin and vanillyl alcohol, the corresponding aldehyde and alcohol, have been identified (Aldrich et al., 1979) in gland secretions of male leaf-footed bugs (Hemiptera: Heteroptera) and vanillin has been identified (Ubik et al., 1975; Vrkoc et al., 1977) as a component of the sex pheromone of the bug *Eurygaster integriceps* (Heteroptera, Scutelleridae). With the present limited capabilities for chemical control of plant-parasitic nematodes (Feldmesser et al., 1985), we are hopeful the report here of the determination of the first structure of a compound with sex pheromone activity in nematodes might lead to development of future novel and environmentally safe control strategies for these pests.

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MATING AND OVIPOSITION BY *Cotesia* (= *Apanteles*)
marginiventris (HYMENOPTERA: BRACONIDAE) IN
PRESENCE OF SYNTHETIC PHEROMONE OF
Spodoptera frugiperda (LEPIDOPTERA:
NOCTUIDAE)

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Abstract—Two blends of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), pheromonal components were evaluated in the laboratory for possible close-range effects on mating and oviposition by *Cotesia marginiventris* (Cresson). Neither a two- nor a five-component blend had any significant effect on mating or parasitization of *S. frugiperda* larvae by this parasitoid. Dosages of 200, 400, and 800 μg were tested.

Key Words—Mating behavior, Parasitoids, *Cotesia* (= *Apanteles*) *marginiventris*, Hymenoptera, Braconidae, *Spodoptera frugiperda*, Lepidoptera, Noctuidae.

INTRODUCTION

Cotesia (= *Apanteles*) *marginiventris* (Cresson) is an important natural enemy of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), because it parasitizes first- and second-instar larvae that die when they reach the fourth instar (Ashley et al., 1982). Also, *C. marginiventris* is one of the most frequently recovered parasitoids from field-collected FAW larvae (Ashley, 1979). Thus, this parasitoid has the potential to reduce substantially FAW larval infestations that cause economic damage to agricultural crops throughout the southeastern and central United States.

Loke et al. (1983) suggested that *C. marginiventris* could be made more

effective through controlled management of its field activity. They reported that the presence of *C. marginiventris* and its searching activity were influenced more by FAW-damaged plants than by the presence of FAW larvae. Loke and Ashley (1984) demonstrated that this parasitoid responds to kairomones from frass of FAW larvae. Parasitization rates were increased 55 and 26% when they confined FAW larvae on filter paper and corn seedlings, respectively, that had been treated with a hexane extract of FAW frass. Nordlund et al. (1983) reported that the host-seeking behavior of *Telenomus remus* Nixon, a parasitoid of FAW eggs, was increased in laboratory and greenhouse bioassays by (Z)-9-tetradecen-1-ol acetate (Z9-14:Ac) and (Z)-9-dodecen-1-ol acetate (Z9-12:Ac), which were originally identified as the components of FAW sex pheromone (Sekul and Sparks, 1967). However, Mitchell et al. (1984) did not detect any significant effect on the level of parasitization of FAW larvae by *Chelonus insularis* Cresson and *Temelucha difficilis* Dasch. when these chemicals were evaporated in 1.1-hectare blocks of field corn. Earlier, Mitchell and McLaughlin (1982) reported significant reductions in FAW mating and oviposition when Z9-14:Ac was applied to a 12-hectare corn field, but Z9-12:Ac, an attractant for FAW but not a component of the FAW pheromone (Tumlinson et al., 1986), was ineffective as a disruptant of pheromone communication among FAW (McLaughlin et al., 1981). Tingle and Mitchell (1982) reported that applications of a four-component mixture of the *Heliothis virescens* (F.) sex pheromone that reduced mating and captures of moths in pheromone-baited traps did not adversely affect parasitization of *H. virescens* larvae in preharvest tobacco. Lewis et al. (1982) determined that the synthetic pheromone of *H. zea* (Boddie) increased the parasitization rate of *H. zea* eggs by *Trichogramma pretiosum* Riley. The study described here was undertaken to observe the close-range effects, if any, of two FAW synthetic pheromonal blends on mating and oviposition by *C. marginiventris* under laboratory conditions.

METHODS AND MATERIALS

The FAW host larvae and *C. marginiventris* used for testing were obtained from our laboratory colonies. Parasitoids and FAW larvae were reared in an environmentally controlled cabinet set at 27°C and 70% relative humidity with a 14-hr photophase. Virgin parasitoids were acquired by placing parasitoid cocoons individually into No. 00 gelatin capsules until eclosion. Upon emergence, females either were confined separately from males or paired with males (3-7 pairs/cage) in Plexiglas cages (25 × 25 × 25 cm) and provided with honey and water. Mating and oviposition tests were conducted 3-8 hr after the beginning of photophase at ambient laboratory conditions (ca. 27°C, 55% relative humidity) in an exhaust hood that was equipped with side and overhead flu-

orescent lighting. The light intensity inside the test chambers was ca. 150 foot-candles when measured with a Weston model 703-60 light meter.

Two blends of FAW pheromonal components were evaluated in both the mating and oviposition tests. One blend consisted of 0.5% (Z)-7-dodecen-1-ol acetate (Z7-12:Ac) and 99.5% (Z9-14:Ac), and the other blend contained five components: 1.0% dodecan-1-ol acetate (S-12:Ac), 0.5% Z7-12:Ac, 0.5% Z9-12:Ac, 79.0% Z9-14:Ac, and 19.0% (Z)-11-hexadecen-1-ol acetate (Z11-16:Ac). Tumlinson et al. (1986) reported that these blends and blends containing three and four components were equally attractive for capturing FAW males in traps; both Z7-12:Ac and Z9-14:Ac were essential for maximum catches.

Rubber septa were used as dispensers for the chemicals. All of the synthetic materials used for the formulation of the pheromone septa were obtained from commercial sources. Each pheromone component was purified by high-performance liquid chromatography on a 25 × 2.5-cm (OD) AgNO₃-coated silica gel column eluted with toluene (Heath et al., 1977). These compounds were then analyzed by capillary gas chromatography on a 50-m × 0.25-mm-ID fused silica CPS-1 (cyanopropyl methyl silicone, Quadrex Corp., New Haven, Connecticut) and a 50-m × 0.33-m-ID fused silica BP-1 (SGE, Austin, Texas). All compounds were determined to be >99% pure.

Dosages of 400 and 800 μg of the two-component blend were evaluated in the mating test, and three dosages (200, 400 and 800 μg) were evaluated in the oviposition test. The 800-μg dosage was used in both tests for bioassaying the five-component blend. In control treatments, septa that had been treated only with the solvent, hexane, were used. During testing, the chemicals were introduced continually into the upper portion of each plastic bioassay chamber by blowing air (300 ml/min) with an aquarium pump through a 1.6-cm-OD brass chamber containing the septum. The release rates of both the two- and five-component blends from the septa containing dosages of 200, 400, and 800 μg were ca. 15, 30, and 60 ng/hr, respectively. Exhaust holes were provided in the bottom of each chamber, and a single, stoppered hole in each top served as an entry for test insects.

In the mating test, four 1-day-old virgin *C. marginiventris* males were first released into a 14.0-cm × 11.5-cm-diam. test chamber and then four virgin females were introduced. The parasitoids were observed during the next 30 min for apparent matings (copulation) or unsuccessful copulation attempts, and data were compared to those in the control. In the oviposition test, 40 first- or second-instar FAW larvae were placed on four pieces of diet (Guy et al., 1985) in the bottom of a 7.0-cm × 9.5-cm-diam. test chamber. Then, one 4- to 7-day-old *C. marginiventris* female that had been confined with males for mating was introduced into the chamber and observed for 20 min for parasitization attempts (contacts with larvae with ovipositor). The parasitoid was then removed, and the FAW larvae were transferred with the diet into a similar container that had

an X-shaped metal grid to provide additional area to reduce larval cannibalism (Loke et al., 1983). Sufficient diet was provided as needed until pupation or emergence of parasitoids.

RESULTS AND DISCUSSION

At the dosages tested, neither the two- nor the five-component FAW pheromone blends had any significant effect on mating by *C. marginiventris* (Table 1). Due to undetermined factors, there were significantly more ($P < 0.05$, Duncan's multiple-range test) apparent matings (5.9 per pair) in the control treatment that was conducted simultaneously with the five-component treatment than in the other control treatments (1.2 and 1.6 matings per pair). However, the mean number of unsuccessful mating attempts (2.0–2.1 per pair) was similar in all of the control treatments. The numbers of apparently successful or unsuccessful matings did not change significantly (unpaired *t*-test) in the treatments with the FAW pheromonal components during the 30-min testing periods. Also, oviposition by *C. marginiventris* was not affected significantly (unpaired *t* test) by the presence of these chemicals (Table 2). During 20-min tests, female parasitoids made 13.0 to 17.8 contacts with FAW larvae in control treatments. These FAW larvae successfully yielded 2.7–5.8 progeny per female parasitoid. There was no significant (unpaired *t* test) difference between control treatments with the solvent (hexane) and treatments with either the two- or five-component FAW pheromonal blends.

TABLE 1. MEAN NUMBER (\pm SE) OF APPARENT MATINGS AND UNSUCCESSFUL MATING ATTEMPTS PER PAIR OF *Cotesia* (= *Apanteles*) *marginiventris* IN PRESENCE OF *Spodoptera frugiperda* PHEROMONE BLENDS

400 μg^a		800 μg^a			
Two-component (6 replications) ^b		Two-component (9 replications) ^b		Five-component (5 replications) ^c	
Control	Treated	Control	Treated	Control	Treated
Apparent matings					
1.2 \pm 0.1	1.1 \pm 0.1	1.6 \pm 0.5	1.9 \pm 0.8	5.9 \pm 1.7	7.6 \pm 1.6
Unsuccessful mating attempts					
2.1 \pm 0.3	2.1 \pm 0.1	2.0 \pm 0.5	1.6 \pm 0.2	2.0 \pm 0.7	1.5 \pm 0.2

^aNo significant effect at $P < 0.05$, unpaired *t* test.

^b0.5% Z7-12: Ac and 95.5% Z9-14: Ac.

^c1.0% S-12: Ac, 0.5% Z7-12: Ac, 0.5% Z9-12: Ac, 79.0% Z9-14: Ac, and 19.0% Z11-16: Ac.

TABLE 2. MEAN NUMBER OF CONTACTS WITH *Spodoptera frugiperda* (FAW) LARVAE AND PROGENY PRODUCED PER *Cotesia* (= *Apanteles*) *marginiventris* FEMALE IN PRESENCE OF FAW PHEROMONE BLENDS

200 μg^a		400 μg^a		800 μg^a			
Two-component (11 replications) ^b		Two-component (13 replications) ^b		Two-component (9 replications) ^b		Five-component (7 replications) ^c	
Control	Treated	Control	Treated	Control	Treated	Control	Treated
Contacts with FAW larvae							
13.0	16.3	16.4	15.2	17.8	11.6	16.9	12.4
± 5.9	± 8.2	± 8.6	± 7.5	± 6.7	± 6.4	± 4.3	± 5.4
Parasitoid progeny produced							
2.9	4.1	5.8	5.3	5.3	3.9	2.7	2.6
± 2.3	± 3.1	± 4.1	± 4.3	± 5.7	± 3.0	± 3.7	± 1.4

^aNo significant effect at $P < 0.05$, unpaired t test.

^b0.5% Z7-12:Ac and 95.5% Z9-14:Ac.

^c1.0% S-12:Ac, 0.5% Z7-12:Ac, 0.5% Z9-12:Ac, 79.0% Z9-14:Ac, and 19.0% Z11-16:Ac.

Although the results of our tests with *C. marginiventris* indicate that the synthetic pheromonal components of its host, FAW, have no effect at close range on parasitism of FAW larvae, the potential value of semiochemicals for modifying insect behavior in pest management systems has been recognized and discussed (Mitchell, 1981, and papers therein; Lewis et al., 1985; Staten et al., 1987). Therefore, the effects that these chemicals may have on both target and nontarget insect species should be investigated, particularly if pheromones or related chemicals are used to disrupt mating and oviposition by the pest species.

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TOXICITY OF ALLELOCHEMICALS FROM WILD
INSECT-RESISTANT TOMATO *Lycopersicon*
hirsutum f. *glabratum* TO *Campoletis*
sonorensis, A PARASITOID OF
Heliothis zea

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Abstract—Greenhouse-grown plants of five tomato lines varying in their level of 2-tridecanone-mediated resistance to *Manduca sexta* (L.) and *Leptinotarsa decemlineata* (Say) were found to adversely affect larvae of *Campoletis sonorensis* (Cameron), a larval endoparasitoid of *Heliothis zea* (Boddie), in a manner directly related to their level of resistance. The parasitoid larvae, which emerge as fifth instars from their host and construct a cocoon on the foliage of their hosts' host plant, suffered extensive mortality during cocoon spinning on highly resistant foliage. Mortality was greatest (82%) on the highly resistant plants of *Lycopersicon hirsutum* f. *glabratum* (accession PI 134417) and an F₁ backcross [(*L. esculentum* × PI 134417) × PI 134417] selection. Mortality was intermediate (40 and 28%, respectively) on backcross selections with moderate and low levels of resistance and least (8%) on susceptible *L. esculentum*. Removal of the glandular trichomes, which contain 2-tridecanone in their tips, from the foliage eliminated differences in parasitoid mortality among plant lines.

Bioassays of 2-tridecanone indicated that it is acutely toxic to fifth instar *C. sonorensis* larvae at the quantities associated with highly resistant foliage and produces symptoms identical to those observed on resistant foliage. 2-Undecanone, a second methyl ketone present in the glandular trichomes of resistant foliage, was also toxic to *C. sonorensis* larvae, but significantly less so than 2-tridecanone. The results support the hypothesis that 2-tridecanone is responsible for the observed mortality of *C. sonorensis* larvae during cocoon construction on resistant foliage.

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Key Words—*Campoletis sonorensis*, Hymenoptera, Ichneumonidae, *Heliothis zea*, Lepidoptera, Noctuidae, 2-tridecanone, trichomes, *Lycopersicon hirsutum* f. *glabratum*, tomato, plant defense, allelochemical.

INTRODUCTION

Accession PI 134417 of the wild tomato species *Lycopersicon hirsutum* f. *glabratum* Mull is highly resistant to a number of arthropod pest species normally attacking the cultivated tomato, *L. esculentum* Mill. (Dimock and Kennedy, 1983; Lin and Trumble, 1986; Lin et al., 1987). High levels of the methyl ketone 2-tridecanone associated with the type VI glandular trichomes of PI 134417 foliage have been shown to confer resistance to *Manduca sexta* (L.) and *Leptinotarsa decemlineata* (Say) (Fery and Kennedy, 1987; Kennedy and Sorenson, 1985; Kennedy et al., 1985). 2-Tridecanone plays only a minor role in the resistance of PI 134417 to *Heliothis zea* (Boddie) (Dimock and Kennedy, 1983; Kennedy, 1986; Kennedy et al., 1987b). Other factors, primarily associated with the foliar lamellae, appear to account for most of the resistance to *H. zea* (Farrar and Kennedy, 1987a).

Recently, Kauffman (1987) demonstrated that parasitism of *H. zea* eggs by *Trichogramma* spp. was reduced from a mean of 71% on *L. esculentum* to 18% on PI 134417. He further demonstrated that the high densities of type VI trichomes on the resistant foliage were responsible for the reduction in egg parasitism.

Larval parasitism of *H. zea* by *Campoletis sonorensis* (Cameron) is also adversely affected on PI 134417 foliage. Kauffman (1987) found, in cage studies, that the incidence of parasitization of *H. zea* larvae by *C. sonorensis* was significantly lower on plant lines possessing high levels of 2-tridecanone-mediated resistance to *M. sexta* than on susceptible lines or those with moderate to low levels of resistance. Fifth-instar *C. sonorensis* larvae kill their hosts, emerge through their host's integument, and spin a cocoon on the foliage of their host's food plant. Kauffman (1987) further found that *C. sonorensis* larvae attempting to construct cocoons on foliage of plant lines containing high levels of 2-tridecanone suffered extensive mortality (94–99% vs. 9–12% on *L. esculentum*). He demonstrated that consumption of 2-tridecanone by *H. zea* larvae parasitized by *C. sonorensis* had no effect on survival of the parasitoid and hypothesized that the mortality observed during cocoon spinning was due to intoxication resulting from direct contact of the fifth-instar parasitoid with 2-tridecanone contained in the tips of type VI trichomes of the resistant foliage. This study was undertaken to test that hypothesis by examining the toxicity of 2-tridecanone to *C. sonorensis*.

METHODS AND MATERIALS

Plant and Insect Rearing. The *C. sonorensis* stock colony was maintained at 27°C and 16:8 light–dark photoperiod on *H. zea* larvae reared on a corn–soya–milk (CSM) diet (Burton, 1970). Parasitoid progeny for our experiments were obtained by exposing late second instars of *H. zea* in lots of 10 for 8 hr to 4-day-old, mated *C. sonorensis* females. Parasitized larvae become immobilized after 10–12 days, shortly before the parasitoid larvae emerge from their host. Immobilized, parasitized larvae were collected for use in experiments. All experiments were conducted in 9 × 1.5-cm plastic Petri dishes with the above laboratory conditions.

Foliage Bioassays. A laboratory experiment tested the hypothesis that the glandular trichomes of resistant foliage do not adversely affect *C. sonorensis* larval survival and cocoon spinning, while a second experiment investigated the effect of removing type VI trichomes on the survival of *C. sonorensis* to adulthood. In both experiments, excised, fully expanded leaves from the upper third of the canopy of glasshouse-grown plants of each of the following five plant lines were used: *L. esculentum* cultivar Walter (susceptible), BC17 {backcross accession [(*L. esculentum* × PI 134417) × PI 134417], low resistance}, BC2 (backcross accession, moderate resistance), BC90 (backcross accession, high resistance), and PI 134417 (*L. hirsutum* f. *glabratum*, high resistance). These levels of 2-tridecanone-based resistance had been previously determined by gas chromatographic (GC) analysis to quantify the 2-tridecanone content of the type VI trichome tips and by bioassay with *M. sexta* (Kauffman, 1987). Where appropriate, glandular trichomes were removed by swabbing the foliage with cotton soaked in Triton X-155 (1 µl/500 ml water) and rinsed thoroughly with distilled water.

H. zea larvae were reared on diet and parasitized as described for the *C. sonorensis* stock colony. After 10–12 days, parasitized larvae become immobilized and take on a pink hue shortly before the parasitoid larva emerges to spin a cocoon and pupate on the foliage. These immobilized, pink, parasitized larvae were placed in groups of five onto the abaxial surfaces of 10–20 leaflets, which completely covered a moistened filter paper disk in the base of a 9 × 2.5-cm Petri dish. Petri dishes were sealed with Parafilm, held at 27°C, and checked on alternate days for proper moisture levels. The experiment was terminated 21 days after initial parasitization, and each parasitized larva was classified as producing one of the following: (1) a *C. sonorensis* larva that died prior to initiation of cocoon construction, (2) a *C. sonorensis* larva that died during the process of cocoon construction, or (3) a complete cocoon with or without an emerged adult. Petri dishes were set up in multiples of 10 *H. zea* (two groups of five larvae each per treatment) per day so that percentages of

each outcome for a given day (rep) were based on the combined data from two Petri dishes (10 larvae) containing similar foliage. The experiment was replicated 10 times for a total of 100 larvae/treatment.

Cocoon spinning by *C. sonorensis* fifth instars on *L. esculentum* and PI 134417 foliage was observed in detail to verify that the larvae were actually disrupting the glandular trichomes on the resistant foliage. The entire cocoon spinning process was observed for 20 larvae on *L. esculentum* and PI 134417 foliage, and for 19 larvae on moderately resistant BC2.

Toxicities of Methyl Ketones. 2-Tridecanone (Fluka Chemical Corp., Hauppauge, New York 11788) and 2-undecanone (Pfaltz and Bauer, Inc., Stamford, Connecticut 06902) were bioassayed to determine their effects on cocoon spinning by *C. sonorensis* larvae. Both compounds were determined to be greater than 99% pure by gas chromatography using a Durabond 15-m, 0.25-mm-ID DB-5 fused silica capillary column with a flame ionization detector. Analyses were run in triplicate using a 10 μ l injection volume, helium as a carrier gas, and a temperature program of 100–275°C increasing at 5°C/min. Acetone solutions covering a range of concentrations were prepared for each methyl ketone. To test each concentration, 0.5 ml of solution was pipetted on to 5.5-cm filter paper disks (Fisher Brand P-5 qualitative) and the solvent allowed to evaporate. Treated filter paper disks were placed in the bottom of a 60 \times 15-mm plastic Petri dish. Five immobilized, pink, parasitized *H. zea* larvae were placed on the filter paper in each dish and the dishes sealed with Parafilm. Fourteen days after initial parasitization of the host, parasitoid larvae were classified as having died prior to completion of cocoon spinning or as having successfully completed cocoon construction.

Concentrations were expressed as micrograms methyl ketone per square centimeter of treated filter paper. Six and eight concentrations of 2-tridecanone (range = 5.35–21.41 μ g/cm²) and 2-undecanone (range = 8.92–53.52 μ g/cm²), respectively, were tested, with each concentration replicated five times (total = 25 larvae/treatment). Controls consisted of confining larvae on acetone-treated filter paper. The number of parasitoid larvae that died following emergence but prior to completion of cocoon spinning was subjected to probit analysis using PROBIT procedure of SAS (SAS User's Guide, 1985) to determine LC₅₀ and LC₉₀ values and the corresponding 95% fiducial limits for each chemical. No mortality was observed in the controls.

Since type VI glandular trichomes of the highly resistant PI 134417 and resistant backcross accessions contain both 2-tridecanone and 2-undecanone, parasitoid larvae emerging from hosts encounter a mixture of these allelochemicals during attempted cocoon construction. Therefore, a second experiment was conducted to determine the effect of a combination of these chemicals on *C. sonorensis* survival. The general procedures were as described for the preceding experiment. A single concentration of 2-undecanone, at a level compa-

able to that found in the PI 134417 plant ($7.78 \mu\text{g}/\text{cm}^2$) was combined with the following three concentrations of 2-tridecanone: 8.75, 13.87, and $44.6 \mu\text{g}/\text{cm}^2$ treated surface. These represent the LC_{25} and LC_{50} values determined in the preceding experiment and the mean level found in the foliage of PI 134417 (Kennedy, 1986), respectively. In addition, each concentration of each chemical alone was bioassayed, and acetone-treated filter paper served as a control. On day 14 following parasitization, percentages of dead *C. sonorensis* larvae were calculated from the 10 parasitized hosts tested within each replicate of each treatment. Each treatment was replicated 10–12 times, and data were subjected to analysis of variance (ANOVA) with mean separation ($P \leq 0.05$) by Duncan's new multiple-range test.

RESULTS AND DISCUSSION

Mortality of fifth-instar *C. sonorensis* larvae during cocoon construction on normal foliage (i.e., with type VI trichomes intact) was greatly affected by tomato genotypes (Table 1). The percentage mortality following emergence of the parasitoid from its host larva but prior to completion of cocoon construction increased with increasing levels of 2-tridecanone-mediated resistance to *M. sexta*. The total mortality experienced by *C. sonorensis* larvae, pupae, and un-emerged adults was highest on the most resistant plant lines (PI 134417 and BC90), intermediate on lines with moderate and low levels of resistance (BC2 and BC17), and least on the susceptible *L. esculentum* cultivar. Kauffman (1987) reported the mean levels of 2-tridecanone in similarly aged plants grown concurrently with those used in this experiment, and under similar conditions in the same greenhouse, to be 1.0, 3.1, 20.1, and $16.5 \mu\text{g}/\text{cm}^2$ of abaxial leaf surface for BC17, BC2, BC90, and PI 134417, respectively. The latter value is considerably below the average of ca. $44 \mu\text{g}/\text{cm}^2$ abaxial leaf surface for PI 134417 reported by Kennedy and Dimock (1983) and observed in other studies (Kennedy, unpublished). We have found only trace amounts of 2-tridecanone associated with *L. esculentum* foliage (Fery and Kennedy, 1987).

Observation of *C. sonorensis* larvae following emergence from their hosts on PI 134417 and *L. esculentum* foliage revealed a rapid and pronounced effect of the PI 134417 foliage on cocoon spinning behavior (Figures 1 and 2). Characteristic weaving motions of the parasitoid's caudal end during cocoon spinning resulted in the rupturing of numerous type VI trichome tips and, hence, contact with the 2-tridecanone and 2-undecanone contained therein. Unlike on *L. esculentum* foliage, on PI 134417 foliage the parasitoid larvae manifested uncharacteristic periods of inactivity that increased in frequency and duration over time. Typically, only the loosely woven outer layers of the cocoons were completed before the larvae became completely moribund. On the PI 134417

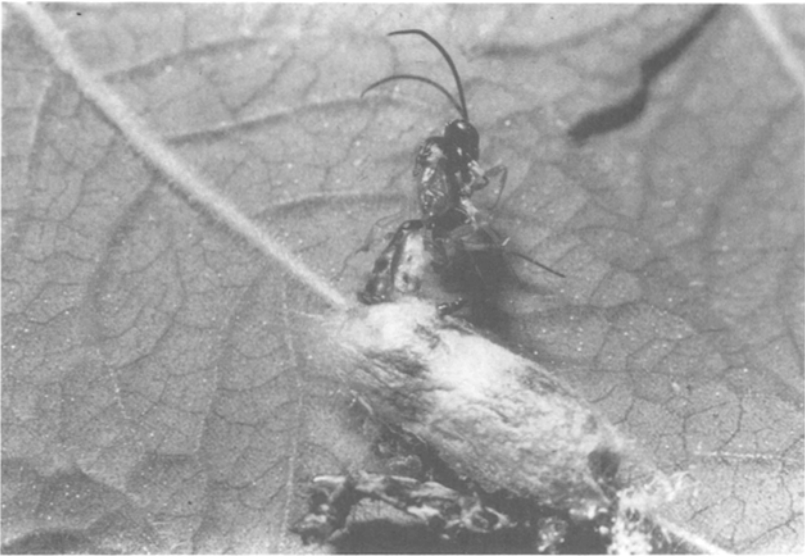


FIG. 1. Complete cocoon and adult of *C. sonorensis* on *L. esculentum* foliage.



FIG. 2. Incomplete cocoon and dead *C. sonorensis* larva on PI 134417 foliage.

foliage, death typically ensued within 2 hr of emergence from the host. However, parasitoid larvae transferred from PI 134417 foliage to untreated filter paper within 30 min of escape from the host were able to construct complete cocoons. On moderately resistant BC2 foliage, a larger proportion of the parasitoids survived to adult emergence (60% vs. 18% on PI 134417). Of those that died, many completed construction of the loose outer layers of the cocoon but died while weaving the tighter inner layers.

Differences in parasitoid mortality among plant lines were eliminated by removal of the type VI trichomes (Table 1). Although this trichome removal experiment was conducted following completion of the experiment involving foliage with intact trichomes, the plants used in both were grown concurrently under similar conditions. Thus, although the results of these experiments are not statistically comparable, they strongly suggest that the plant effects on *C. sonorensis* larvae are mediated through the glandular trichomes, which are known to contain significant quantities of the methyl ketones 2-tridecanone and 2-undecanone. These ketones are toxic to a number of insect species (Williams et al., 1980; Dimock et al., 1982; Kennedy and Sorenson, 1985; Farrar and Kennedy, 1987b; Lin et al., 1987).

Results of our bioassays of these methyl ketones indicate they are toxic to fifth-instar *C. sonorensis* larvae as well. In the probit analyses, the tests for heterogeneity gave chi-square values that were nonsignificant for both 2-tridecanone and 2-undecanone ($\chi^2 = 3.134$ and 3.237 , respectively; probability of greater $\chi^2 > 0.35$ for both). The LC_{50} and LC_{90} values indicate that 2-tridecanone is approximately three times more toxic to *C. sonorensis* larvae than is 2-undecanone (Table 2). These values for 2-tridecanone represent only 31 and

TABLE 1. MORTALITY OF *C. sonorensis* PROGENY WHEN PLACED ON FOLIAGE OF VARIOUS PLANT LINES AS SUBSTRATE FOR COCOON SPINNING^a

Plant line	Foliar trichomes intact		Foliar trichomes removed	
	Dead larvae ^b	Total dead ^c	Dead larvae ^b	Total dead ^c
<i>L. esculentum</i>	1.1 a	7.7 a	3.2	10.8
BC17	9.6 ab	27.9 b	12.1	25.6
BC2	17.1 b	40.2 b	18.2	28.6
BC90	75.9 c	81.8 c	21.5	32.5
PI 134417	74.9 c	82.2 c	9.4	22.5
			NS	NS

^aValues are \bar{X} percent mortality; 10 replications; ANOVA ($P \leq 0.05$) conducted using Duncan's data transformed to arcsin percent new multiple-range test.

^bIncludes all larvae that died prior to completing cocoon construction.

^cIncludes all larvae, pupae, and unemerged adults.

TABLE 2. TOXICITY OF 2-TRIDECANONE AND 2-UNDECANONE TO LARVAE OF *Campoletis sonorensis*^a

Treated filter paper	LC ₅₀	LC ₉₀
2-Tridecanone	13.9 (10.08, 17.95)	23.6 (19.07, 38.80)
2-Undecanone	38.9 (32.34, 47.79)	69.4 (57.32, 97.81)

^aLC₅₀ and LC₉₀ ± 95% fiducial intervals expressed as µg/cm² treated surface; data are based on 25 parasitized hosts in five replications.

53%, respectively, of the 44.6 µg of 2-tridecanone per cm² of PI 134417 foliage reported by Kennedy and Dimock (1983), but 84 and 143%, respectively, of the 2-tridecanone levels found in PI 134417 foliage grown concurrently with the foliage used in this study (Kauffman, 1987). Bioassays of mixtures of 2-tridecanone and 2-undecanone provided no evidence for synergism in toxicity to *C. sonorensis* (Table 3). This is in contrast to the synergism observed in toxicity of the mixture to the lepidopterous species *H. zea* (Farrar and Kennedy, 1987b), *Spodoptera exigua* and *Keiferia lycopersicella* (Lin et al., 1987).

Our results support the hypothesis that 2-tridecanone is responsible for the observed mortality of *C. sonorensis* larvae on the resistant foliage. First, par-

TABLE 3. MORTALITY OF *C. sonorensis* LARVAE ON CHEMICALLY TREATED FILTER PAPER

Chemical (µg/cm ²)	Representation of level	Total number of parasitoid larvae	Mortality, % (± SD) ^a
Acetone	Control	114	9.1 (11.09) a
2-Undecanone 7.78	PI 134417	118	27.2 (17.88) b
2-Tridecanone 8.75	LC ₂₅	120	25.8 (18.81) b
2-Tridecanone 13.87	LC ₅₀	118	68.4 (17.81) d
2-Tridecanone 44.6	PI 134417	100	100.0 (0) e
2-Tridecanone 8.75 + 2-Undecanone 7.78	LC ₂₅ + PI 134417	120	45.8 (20.21) c
2-Tridecanone 13.85 + 2-Undecanone 7.78	LC ₅₀ + PI 134417	112	62.5 (16.54) cd
2-Tridecanone 44.6 + 2-Undecanone 7.78	PI 134417 + PI 134417	98	98.9 (3.51) e

^aData are the means of 10-12 replications; analysis of variance (ANOVA) and vertical separation of means ($P \leq 0.05$, DNMR).

asitoid mortality on foliage of the various plant lines was positively associated with their level of 2-tridecanone-mediated resistance to *M. sexta*. Second, removal of the 2-tridecanone-containing glandular trichomes from the foliage eliminated the detrimental effects of the various plant lines on *C. sonorensis*. Finally, the symptoms of intoxication manifested by *C. sonorensis* larvae in response to levels of 2-tridecanone on filter paper similar to those associated with PI 134417 foliage were identical to those manifested on PI 134417 foliage.

Although the toxicity of 2-tridecanone to *C. sonorensis* and *H. zea* is similar [$LC_{50} = 13.9$ and $17.1 \mu\text{g}/\text{cm}^2$ treated surface, respectively (Dimock et al., 1982)] in treated filter paper bioassays of the type used in this study, on resistant foliage *C. sonorensis* is affected to a much greater degree than its host, *H. zea*. Whereas 82% mortality was observed among *C. sonorensis* spinning cocoons on PI 134417 foliage, only about 20% of neonate *H. zea* larvae placed on or hatching from eggs incubated on PI 134417 foliage die. Many of the remaining larvae manifest symptoms of acute toxicity (convulsions, paralysis) but subsequently recover. Unlike fifth-instar *C. sonorensis* larvae, which disrupt numerous type VI trichomes and directly contact the 2-tridecanone contained therein, neonate *H. zea* are very small and rarely discharge type VI trichome tips before receiving a sublethal but narcotizing dose of 2-tridecanone from the fumes that surround the resistant foliage. These narcotized larvae subsequently recover, apparently completely, and are able to tolerate exposure to much higher levels of 2-tridecanone with no apparent ill effects (Dimock and Kennedy, 1983; Kennedy, 1984). This recovery appears to be related to the induction of elevated levels of cytochrome P-450, which results from exposure of *H. zea* eggs or larvae to 2-tridecanone or to PI 134417 foliage (Kennedy et al., 1987a).

The differential effects of 2-tridecanone-mediated resistance to *M. sexta* and *L. decemlineata* on *C. sonorensis* and *H. zea* are such that the suppressive effects of the parasitoid on the *H. zea* larval population will be reduced relative to those observed on tomato lines lacking 2-tridecanone-mediated resistance. In addition to a reduced level of parasitization of *H. zea* larvae by *C. sonorensis* on resistant foliage (Kauffman, 1987), few parasitoid larvae emerging from hosts and attempting to pupate on 2-tridecanone-laden foliage will survive to adult, thus precluding a population response by *C. sonorensis* to increases in *H. zea* larval populations on *M. sexta* resistant tomato lines. In areas where *C. sonorensis* is important in the natural biological control of *H. zea*, the use of 2-tridecanone-mediated resistance to *M. sexta* and *L. decemlineata* would be undesirable.

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PATTERN OF VOLATILE COMPOUNDS IN DOMINANT AND SUBORDINATE MALE MOUSE URINE

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Abstract—The urinary volatiles from dominant and subordinate male mice were chromatographically compared, both immediately and seven days after dominant-subordinate relationships between pairs were formed. Statistical comparison of the peak areas of volatile constituents present in male urine revealed that 16 urinary compounds exhibited substantial concentration differences depending upon social status of the animals. Urinary dihydrofurans, ketones, and acetates exhibited a significant, long-term (seven-day) decrease in the urine of subordinates when compared to control and dominant males. Two sesquiterpenic compounds, α - and β -farnesene, were elevated only in the dominant urine one week after dominance was established. 2-(*sec*-Butyl)-4,5-dihydrothiazole was found in higher concentration in bladder or excreted urine of dominant males when compared to subordinates. Of the 16 compounds subjected to statistical analysis, four exhibited hormonal dependency: α - and β -farnesene, dehydro-*exo*-brevicommin, and 2-(*sec*-butyl)-4,5-dihydrothiazole. Neither dehydro-*exo*-brevicommin nor 2-(*sec*-butyl)-4,5-dihydrothiazole was present in the urine of immature and castrated males. Testosterone treatment restores their presence in male urine. Also, α - and β -farnesene were absent in the urine of immature males and significantly reduced in the urine of castrated males. The absence of α - and β -farnesene in bladder urine suggests that one of the sex-accessory glands may be the site of their origin.

Key Words—Pheromones, urinary volatiles, capillary gas chromatography, house mouse, social status.

INTRODUCTION

Mouse urine has been shown to be a rich source of olfactory cues that elicit changes in the reproductive behavior and physiology of recipient animals (Bronson, 1974). The male urinary pheromones induce the pregnancy block, estrus

synchronization, puberty acceleration, sexual attraction, and intermale aggression. These pheromones are androgen-dependent (Bronson and Whitten, 1968) and are not present in the urine of castrated mice (Bruce, 1965). Social subordination suppresses gonadal function in male laboratory mice (Bronson, 1973) and brown lemmings (Huck *et al.*, 1981), inhibits scent marking (Desjardins *et al.*, 1973), suppresses the ability of male urine to accelerate the onset of puberty in females (Lombardi and Vandenberg, 1977), and decreases the ability of the urine to cause pregnancy blockage (Huck, 1982).

Male mouse urine contains well over 100 volatile components; however, only a small number of these show a dependency on the male hormones (Novotny *et al.*, 1980). During our investigation of urinary volatiles by high-resolution, gas-phase analytical techniques, we observed that castration eliminates 2-(*sec*-butyl)-4,5-dihydrothiazole and dehydro-*exo*-brevicommin in male urine (Schwende, 1982; Novotny *et al.*, 1980). Testosterone supplementation can renew the normal concentrations of these compounds in castrate male urine (Novotny *et al.*, 1984). Both substances have been synthesized (Prodan, 1986; Wiesler *et al.*, 1982).

Studies of the behavioral activity of 2-(*sec*-butyl)-4,5-dihydrothiazole and dehydro-*exo*-brevicommin revealed that they elicit vigorous and persistent antagonistic behavior from male mice (Novotny *et al.*, 1985). When both compounds are added to castrate male urine, they create an olfactory signal that attracts females (Jemiolo *et al.*, 1985) and has the ability to abolish the suppressive effect of high caging density on the estrous cycle (Jemiolo *et al.*, 1986).

While it has long been assumed that urinary constituents, in a qualitative or quantitative sense, may signal the dominance status of male rodents, no chemical data on the subject have been reported at this date. The goal of the present study was to quantify urinary volatile constituents under various social conditions and rationalize these findings with respect to hormonal processes.

METHODS AND MATERIALS

The ICR/Alb albino mice used in the experiments were initially mated pairs purchased from Ward's Natural Science Establishment, Inc. (Rochester, New York), which were then randomly bred closed-colony. All animals were maintained at $21 \pm 0.2^\circ\text{C}$, 50–70% humidity, and a 12-hr light/12-hr dark daily regime (lights on at 0600 hr). Purina mouse chow and water were supplied *ad libitum* throughout the experiment. Bedding was changed weekly.

Prior to the social rank test, each male was maintained in social isolation from 25 days of age. At the time of the experiment, the animals were 90–100 days of age. The control urine (1 ml) was collected from each male prior to beginning the rank test. Next, dominance or subordination was established by

pairing two males in a neutral arena for 10 min on each of 10 consecutive days. Males were originally paired so that neither male weighed over 2 g more than his partner. An animal was classified as dominant if its daily chase-attack score was significantly higher (chi-square test; employing a 0.01 confidence interval) than that of the other member of the pair. Only males displaying a stable dominant-subordinate relationship during the 10 ranking tests were used. Eleven dominant and 11 subordinate males served as urine donors.

On the first day after completion of the social test, the first collection (collection 1) of urine from dominant and subordinate males took place. After first collection, the animals were kept in the home cage, undisturbed for the next six days. The second collection (collection 2) took place seven days after the social rank test was finished. One milliliter of urine was collected from each dominant and subordinate male during both collection periods. Two dominants and two subordinates served as bladder urine donors. Urine was collected directly from the bladder using needle and syringe. Two samples of bladder urine (1 ml of each) from dominant and subordinate males were analyzed. Subsequently, all tested animals were sacrificed, and the body, adrenal, and preputial glands, the seminal vesicle plus coagulating glands, and testes were dissected out and weighed.

Urine samples were also collected from immature and castrated males, as well as the castrates with testosterone. Three 1-ml urine samples were collected from 21 immature males when they were 21 days of age. In addition, nine immature males were castrated and housed in a group of three per cage until they reached the age of 90 days. At this time, three 1-ml samples of castrate urine were collected over three consecutive days. Four additional 21-day-old males were castrated and housed in a group of two per cage. At the age of 90 days, a testosterone capsule (4 mm in size) was implanted in each animal, using the technique described by Moore (1981) and Boyer *et al.* (1988). Ten days later, 4 ml of urine was collected from these testosterone-treated animals.

For a single chromatographic analysis, 1-ml urine samples were used. The number of chromatographic analyses for each type of urine corresponded with the number of milliliters for collected urine.

All urine samples were collected on a block of Dry Ice and immediately stored at -20°C . Each collection period lasted 6 hr and started the same time each day, *i. e.*, from 0900 to 1500 hr.

The urinary volatiles of all investigated samples were analyzed by capillary gas chromatography and identified by combined capillary gas chromatography-mass spectrometry, following the procedures described by Novotny *et al.* (1974) and Schwende *et al.* (1986). Additional verification of the chemical identity was based on methane and isobutane chemical ionization spectra. The identities of the sesquiterpenes were further confirmed by coinjections of the authentic compounds. *E,E*- α -Farnesene was synthesized (Negishi and Matsushita, 1984),

and *E*- β -farnesene was isolated from the oil of chamomile (Murray, 1969; Sorm *et al.*, 1949, 1951).

Statistical comparisons of the concentrations of excreted volatiles were made using one-way analysis of variance (ANOVA; *F* at $P < 0.05$) with post-hoc comparison by Duncan's new multiple-range test (*t* at $P < 0.02$) (Zar, 1974).

RESULTS AND DISCUSSION

The design of the experiment reported here was based on a series of our observations indicating that the concentrations of certain urinary volatile metabolites in male mouse urine were changed in response to testosterone levels (Novotny *et al.*, 1980). The observation of some authors that dominant animals tend to have higher levels of testosterone than subordinates (McKinney and Desjardins, 1973; Edwards and Rowe, 1975; Rose *et al.*, 1975) provided further incentive for this study. Laboratory evidence also indicates that male mouse urine has an odor indicative of social status (Jones and Nowell, 1973a). The above information has led to the present investigations of the urinary volatiles of trained fighters and subordinates.

The capillary gas chromatograms shown in Figure 1 are representative of the substance profiles obtained from bladder and externally voided urine of male mice. The list of compounds identified in these profiles is provided in Table 1. Sixteen volatiles exhibited statistically significant ($P < 0.02$) changes in concentration, depending on social status of animals or their endocrinological conditions. They have been subsequently identified as various dihydrofurans, ketones, acetates, dehydro-*exo*-brevicomine, 2-(*sec*-butyl)-4,5-dihydrothiazole, and the sesquiterpenes, α - and β -farnesene.

As seen in Figure 2A, the concentration of all selected dihydrofurans, ketones (except peak 10), and acetates are drastically decreased in the urine of subordinates collected immediately after social rank test (collection 1). Maintenance of all these volatiles at very low concentrations was still observed in the urines collected a week after the social rank test was completed (collection 2).

Urine of dominant males contained the dihydrofurans and acetates in concentrations similar to control animals, regardless of the time of collection (Figure 2B). A significant decrease of urinary ketones was observed only for peaks 7, 8, and 11, during either the first or second collection. While peak 10 exhibited no change of concentration in subordinate urine, it showed a trend to increase its level in dominant urine during both periods of collection.

The concentrations of α - and β -farnesene dropped significantly in both dominant and subordinate urine immediately after the social rank test (collection

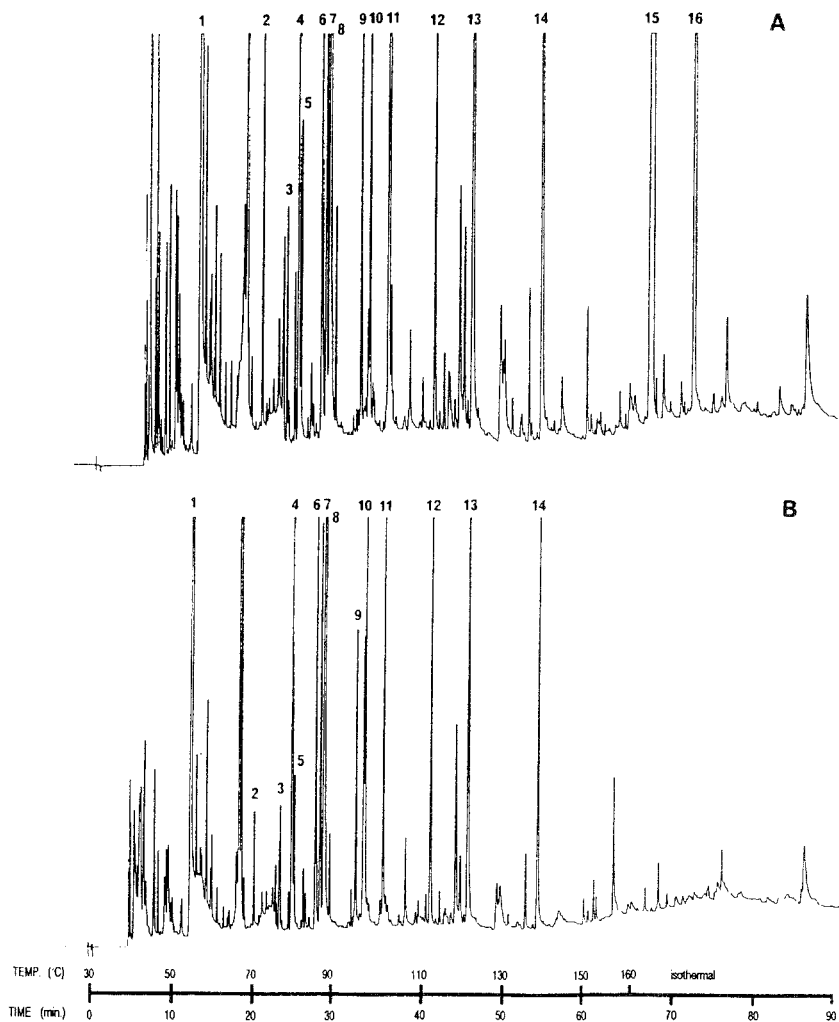


FIG. 1. Gas-chromatographic profiles of (A) externally voided dominant male urine and (B) bladder urine of a subordinate male.

1; Figure 2A,B). Only in dominant urine obtained during the second collection did both sesquiterpenes show a dramatic increase in concentration when compared to control and subordinate animals (Figure 2A,B).

Social status of the males did not influence the dehydro-*exo*-brevicommin concentration (Figure 2A,B). However, the concentration of 2-(*sec*-butyl)-4,5-dihydrothiazole dropped significantly in the urines of subordinates during both

TABLE 1. VOLATILE COMPOUNDS IN URINE OF ICR/Alb MALE MICE

Class of compounds	Structure	Peak number
Dihydrofurans	mol wt 126 ^a	1
	mol wt 126 ^a	2
	m.w. 126 ^a	3
Ketones	2-Heptanone	4
	5-Heptene-2-one	7
	4-Heptene-2-one	8
	3-Heptene-2-one	9
	6-Methyl-6-hepten-3-one	10
	6-Methyl-5-hepten-3-one	11
	Acetophenone	14
Acetates	<i>n</i> -Pentyl acetate	5
	2-Penten-1-yl acetate	6
Dehydro- <i>exo</i> -brevicommin		12
2-(<i>sec</i> -butyl)-4,5-dihydrothiazole		13
Sesquiterpenes	β -Farnesene	15
	α -Farnesene	16

^a Presumed isomeric cyclic vinyl ethers unique to the mouse; dehydration products of a known 5,5-dimethyl-2-ethyltetrahydrofuran-2-ol.

collection periods (Figure 2A). The low level of this substance in dominant male urine was not found till some time after the social rank test (Figure 2B).

Our detailed chemical investigations of urine from immature males, adult intact males, castrate males, and castrated animals with testosterone implants clearly indicate that four of the selected volatile compounds are the most interesting with respect to hormonal function and social status of mice. These compounds are α -farnesene, β -farnesene, dehydro-*exo*-brevicommin and 2-(*sec*-butyl)-4,5-dihydrothiazole (Table 2). None of these four compounds were observed in the urine of immature males. The remaining 12 of the 16 selected compounds were always present in the urine from immature males, although at a low concentration. Castration reduced the levels of α - and β -farnesene and completely depressed the concentrations of dehydro-*exo*-brevicommin and 2-(*sec*-butyl)-4,5-dihydrothiazole (Table 2). Ten days of testosterone treatment reduced the levels of both sesquiterpenes in the male urine, when compared to intact and castrated males, and partially restored the presence of dehydro-*exo*-brevicommin and 2-(*sec*-butyl)-4,5-dihydrothiazole (Table 2). The decrease of α - and β -farnesene to the value below 1.0 (arbitrary units) was previously observed only for subordinate male urine at collection 1 (β -farnesene = 0.4 ± 0.2 , and α -farnesene = 0.7 ± 0.4).

Dehydro-*exo*-brevicommin was found at a similar concentration in both

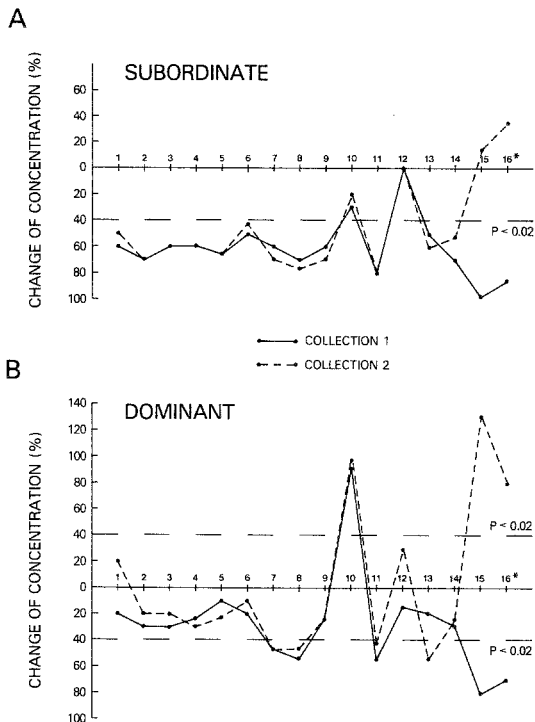


FIG. 2. (A and B). Effect of social status and the time of urine collection on the levels of urinary volatile constituents. Percentage differences in the peak areas of urinary dihydrofurans (peaks 1–3), acetates (peaks 5 and 6), ketones (peaks 4, 7–11, and 14), dehydro-*exo*-brevicomine (peak 12), 2-(*sec*-butyl)-4,5-dihydrothiazole (peak 13), and sesquiterpenes (peaks 15 and 16) are seen between collection 1 and collection 2 for (A) subordinate and (B) dominant males.

dominant and subordinate bladder urines, although at a significantly higher level when compared to control, excreted urine. 2-(*sec*-Butyl)-4,5-dihydrothiazole was present in the bladder urine of subordinate and dominant males at concentrations comparable to those found in excreted urine, but dominant bladder urine contained significantly more 2-(*sec*-butyl)-4,5-dihydrothiazole than did the bladder urine of subordinates (Table 2).

The bladder urine of neither dominant nor subordinate males contained α - and β -farnesene (Figure 1; Table 2).

There were no significant differences in the body weights and the weights of seminal vesicles plus coagulation glands between control, dominant, and subordinate males (Table 3). In marked contrast, the dominant animals had significantly greater preputial glands when compared to subordinate and control

TABLE 2. MEAN (\pm SEM) VALUE OF PEAK AREAS FOR FOUR SELECTED VOLATILE COMPOUNDS OF MALE MOUSE URINE AFTER HORMONAL MANIPULATIONS AND SOCIAL RANK TEST

Type of sample	Peak area in arbitrary units ^a			
	β -farnesene [15]	α -farnesene [16]	dehydro- <i>exo</i> - brevicomin [12]	2-(<i>sec</i> -butyl)-4,5- dihydrothiazole [13]
Excreted urine				
Immature	0.0	0.0	0.0	0.0
Intact	12.8 (1.3)a	5.2 (0.4)a	4.2 (0.4)a	18.6 (1.9)a, c
Castrated (CD)	3.1 (1.3)b	1.2 (0.4)b	0.0	0.0
CD + testosterone	0.6 (0.0)c	0.3 (0.0)b	2.1 (0.3)b	0.4 (0.0)b
Bladder urine				
Dominant	0.0	0.0	7.9 (0.5)c	25.1 (2.3)a
Subordinate	0.0	0.0	6.3 (0.7)c	11.6 (1.9)c

^aPeak numbers are shown in brackets. The means not marked by letters (a, b, c) are significantly different at the 0.02 level (ANOVA).

males. Only subordinate males had significantly larger adrenal glands and smaller testes when compared to control and dominant males (Table 3).

Significant, long-term decreases of urinary dihydrofurans, ketones, acetates, and 2-(*sec*-butyl)-4,5-dihydrothiazole in subordinate urine and only a slight decrease of these same compounds in the urine from dominant males may

TABLE 3. BODY WEIGHTS (g) AND RELATIVE ORGAN WEIGHTS (mg/100 g) OF MICE EXPERIENCING REPEATED (10-DAY PERIOD) VICTORY OR DEFEAT^a

	Control ^b (N = 10)	Subordinate (N = 12)	Dominant (N = 12)	Analysis of Variance
Body	39.7 (1.3) ^c	37.4 (0.9)	37.5 (0.7)	NS
Paired testes	777.1 (23.7)	637.4 (52.1)a	778.2 (37.1)	P < 0.05
Paired seminal vesicles and coagulating glands	849.7 (25.7)	888.0 (37.6)	938.8 (46.5)	NS
Paired preputial glands	285.9 (20.6)a	332.1 (23.1)b	434.6 (16.5)c	P < 0.001
Paired adrenal glands	8.9 (0.7)	14.4 (0.9)a	8.6 (0.9)	P < 0.001

^aBody and listed organs were weighed 7 days after the last fight took place.

^bSocially inexperienced, singly caged males that were sacrificed at 6 months of age.

^c \pm SEM values are given in parentheses.

reflect the circulation of FSH, LH, and corticosteroids in the plasma. Both FSH and LH were found to be depressed in the plasma of dominant and subordinate male mice after initial pairwise fights (Bronson, 1973). According to Bronson *et al.* (1973), FSH returned to the control level after several days in the dominant males, but remained depressed in the subordinate males. LH showed periodic, fluctuating circulation in the plasma of both dominant and subordinate animals.

The absence of α - and β -farnesene in bladder urine suggests that these sesquiterpenes may originate from the sex-accessory glands. The preputial glands have long been considered a possible site for the production of aggression and sex-related signals (Aron, 1979; Caroom and Bronson, 1971). Territorial marking is certainly of paramount importance for the establishment of social and/or sexual dominance. Territorial marking may be associated with aversive properties of the marker. It has been shown that the urine from dominant males possessed greater potency than that from subordinate males in aversion trials (Jones and Nowell, 1973a,b). The androgen-dependent aversive pheromones were shown to be absent in bladder urine (Jones and Nowell, 1973a,b, 1974). Testosterone treatment restored the aversive efficacy of urine in castrated males with a five-day latency, thus suggesting the involvement of some androgen-dependent tissue rather than that of an androgen-excreted metabolite (Jones and Nowell, 1974; Mugford and Nowell, 1970).

Since dehydro-*exo*-brevicommin and 2-(*sec*-butyl)-4,5-dihydrothiazole are clearly present in the bladder urine, we do not expect these compounds to be the aversion-eliciting compounds. We know that these two substances, when added to castrate urine, are sufficient to elicit increased aggressiveness of trained fighters (Novotny *et al.*, 1985). Interestingly, we find that 2-(*sec*-butyl)-4,5-dihydrothiazole has more than double the concentration in dominant bladder urine when compared to subordinate bladder urine (Table 2). The addition of preputial gland secretion to bladder urine is reported to induce further aggressiveness of fighter males (Jones and Nowell, 1973b). It is thus possible that α - and β -farnesene are the constituents that potentiate the known effectiveness of dehydro-*exo*-brevicommin and 2-(*sec*-butyl)-4,5-dihydrothiazole in eliciting aggression.

In agreement with observations by others (Bronson and Eleftheriou, 1964; Bronson, 1973; Lloyd, 1972), dominant males from our experiment exhibited larger preputial glands than did subordinates. The increase in concentrations of α - and β -farnesene that was established one week after dominance thus coincides with the increase in the weight of preputial glands. The low concentration or absence of these compounds in castrate or immature male urine might support the notion that production of these sesquiterpenes is androgen-dependent.

A significant decrease of α - and β -farnesene in castrated males with testosterone supplementation could have been caused by surgical stress or occa-

sional stressful encounters between castrated animals. The constant release of testosterone from the capsule for a period of 10 days and social interaction between testosterone-treated castrates could have a similar effect on the production of α - and β -farnesene to that observed for intact males (after the rank test was finished), when both sesquiterpenes dropped significantly in both dominant and subordinate males.

It is interesting that α - and β -farnesene are known in nature to elicit a variety of responses in insects (Bowers *et al.*, 1972; Edwards *et al.*, 1973; Vander Meer *et al.*, 1981). Based on the chemical data presented here, extensive behavioral testing of these terpenes in mice should be a subject of future studies.

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EUPATORIOCHROMENE AND ENCECALIN, PLANT GROWTH REGULATORS FROM YELLOW STARHISTLE (*Centaurea solstitialis* L.)

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Abstract—Two chromenes, eupatoriochromene (1) and encecalin (2), have been isolated from yellow starthistle (*Centaurea solstitialis* L.). Both chromenes retard seed germination and reduce radicle and hypocotyl growth of weed and crop plant seedlings. In addition, 1 increases adventitious root formation of mung bean cuttings.

Key Words—Yellow starthistle, *Centaurea solstitialis*, chromenes, eupatoriochromene, encecalin, benzopyrans, allelopathy, plant growth regulator.

INTRODUCTION

Yellow starthistle (*Centaurea solstitialis* L.) is an aggressive noxious weed in the western United States and other parts of the world (Koehler, 1965; Sunderland, 1976; Maddox, 1981; Maddox et al., 1985; Maddox and Mayfield, 1985). Maddox et al. (1985) showed that the plant germinates during the winter rains and continues to grow and is plentiful until spring rain ceases. As soil moisture decreases, mortality increases, but even in the summer droughts of California, yellow starthistle is abundant. This annual herb spreads rapidly and, like many *Centaurea* spp., it is suspected of being allelopathic. *C. solstitialis* has been reported to contain alkaloids (Mamedov, 1956), polyphenols (Masso et al., 1979; Kamanzi and Raynaud, 1976), terpenoids (Cassady and Hokanson, 1978; Buttery et al., 1986), and sesquiterpene lactones (Theissen et al., 1969; Zarghami and Heinz, 1969; Mukhametzhonov et al., 1972; Cassady et al., 1979; Merrill and Stevens, 1985). This paper reports the first isolation from a *Cen-*

taurea spp. of the two chromenes, eupatoriochromene (**1**, desmethylencecalin, 2,2-dimethyl-6-acetoxy-7-hydroxybenzopyran, EUP) and encecalin (**2**, 2,2-dimethyl-6-acetoxy-7-methoxybenzopyran, ENC) (Figure 1).

Chromenes have been previously isolated from some members of the Asteraceae family (Bjeldanes and Geissman, 1969; Bohlmann and Grenz, 1970, 1977; Hegnauer, 1977; Wisdom and Rodriguez, 1982; Klocke et al., 1985; Proksch and Rodriguez, 1983, and references therein). They have been reported to be insecticidal (Proksch et al., 1983; Klocke et al., 1985; Wisdom et al., 1983), and they exhibit antifeedant (Wisdom et al., 1983) and antijuvenile hormone (Bowers et al., 1976) activity in insects. However, none have been reported to have plant growth regulatory activity. This paper reports the plant growth regulatory activity of eupatoriochromene (**1**) and encecalin (**2**).

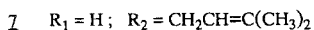
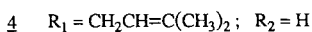
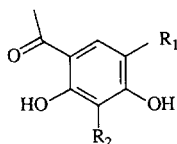
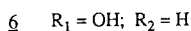
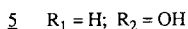
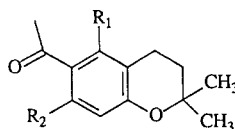
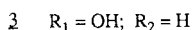
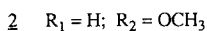
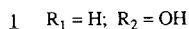
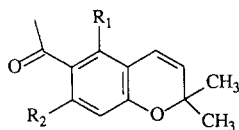


FIG. 1. Structures of compounds **1-7**.

METHODS AND MATERIALS

Plant Material. Aerial parts of yellow starthistle were mowed, dried, and baled at the University of California at Davis, California. The air-dried plant was then ground in a hammermill through a $\frac{1}{8}$ -in. screen.

Isolation and Identification. The ground plant material was extracted in a large Soxhlet extractor with distilled hexanes for two days. After evaporation of solvent, the dark oily residue was then partitioned between petroleum ether, bp 40–60° (Skelly F)–benzene (1:1) and methanol–H₂O (4:1). The aqueous layer was concentrated *in vacuo* and chromatographed on silica gel column, eluting with Skelly F–ether (1:1). The first fraction was repeatedly chromatographed on a rotary preparative thin-layer chromatotron (Harrison Research) using Skelly F–ether (5:1) to give the two chromenes, eupatoriochromene (1) and enecalin (2). The chromenes were synthesized according to a procedure by Steelink and Marshall (1979). Physical properties (¹H]- and [¹³C]NMR, UV, MS, GC, and TLC) of the isolates were identical to those of the synthetic chromenes.

Seed Germination Bioassay. Twenty-five lettuce seeds (Black Seeded Simpson) were placed in Petri dishes containing 20 ml of 0.5% agar (Bacto-agar, Difco Laboratories, Detroit, Michigan) using 10, 50, and/or 100 ppm of test compound. All the test compounds had limited solubility in H₂O and 50 or 100 ppm was above saturation point. Seeds were incubated in the dark at 20–23°C and counted after three days. Seeds were considered germinated when the skin was broken and the radicle was greater than 1 mm long. Duplicates were run for each sample.

The effect of EUP and ENC on seed germination of red millet (*Panicum miliaceum*), alfalfa (*Medicago sativa*), barnyard grass (*Echinochloa crusgalli*), cucumber (*Cucumis sativus*, cv. Sumter), perennial ryegrass (*Lolium perenne* L.), and yellow starthistle was tested in a similar manner, but using 50 seeds per Petri dish in 50- and 100-ppm test solutions, respectively, and counted after three days. Where there was some effect, the dishes were read again after six days.

Seedling Growth Bioassay. For procedure A, the effect of test compound on radicle and hypocotyl growth of lettuce seedlings was tested at 0, 5, 10, 20, 40, 60, 80, 100, and 200 ppm in 20 ml of 0.5% agar solution. The appropriate amount of compound in ether was placed in each Petri dish. After ether was added to make 0.5 ml, the solvent was evaporated. Hot agar solution was added. The dish was heated on a hot plate to dissolve compounds, stirred, and cooled to room temperature. Twenty seeds were placed in each Petri dish containing test compound and grown in the dark at 20–23°C for three days. The lengths of the radicle and hypocotyl were measured to the nearest millimeter. Duplicates were run for each concentration.

For procedure B, the effect of some of the compounds on radicle and hypocotyl growth of barnyard grass, red millet, alfalfa, cucumber, yellow starthistle, green foxtail (*Setaria viridis*), annual morning glory [*Ipomoea purpurea* (L.) Roth] and soybean [*Glycine max* (L) Merr., cv. Lakota] was tested in a similar manner, but using pregerminated seedlings. Seeds were germinated to 1–3 mm on 0.5% agar in the dark at 20–23°C. Seedlings of equal length were then transferred into Petri dishes containing test compounds and grown as before for three to five days.

Mung Bean Cutting Bioassay. Mung bean (*Phaseolus aureus* Roxb) seeds were sterilized and grown in sterile soil for seven days in a growth chamber with a 16-hr day at 23°C and 8-hr night at 18°C. Cuttings were made 2½–3 cm below cotyledons and placed in vials containing 10 ml of distilled water, 100 µl acetone, and 0, 10, 20, 40, or 80 ppm eupatoriochromene. Two cuttings were placed in each vial. Three replicates of each concentration were made. Distilled water was added to each vial every 24 hr to maintain 10 ml. After 12 days in the growth chamber, the number of roots were counted and averaged over the replicates. A similar bioassay, but without acetone, was also done in duplicate.

RESULTS AND DISCUSSION

Seed Germination. The effect of eupatoriochromene, enecalin, and their precursors **4** and **5** and isomeric chromene **3** and its precursors **6** and **7** was tested on the germination of lettuce seeds. Eupatoriochromene inhibits germination at 100 ppm and enecalin at 10 ppm or above (Table 1). Compounds **3–7** do not retard germination at 100 ppm. The apparent inhibitory effect of chromenes **1** and **2** thus cannot be attributable to their precursors or to the chromene ring alone.

Germination of other seeds was also tested (Table 1). Germination of alfalfa and cucumber is not inhibited by either eupatoriochromene or enecalin at 100 ppm, but barnyard grass is slightly retarded at that concentration in ENC. In addition, red millet and perennial ryegrass are both inhibited at 50 ppm (three days). In each case where inhibition is observed, ENC is more effective than EUP. The initial inhibitory effect observed in red millet and barnyard grass, however, is reduced with increasing time until germination is equal (six days), within experimental error, to that of control. The effect of enecalin and eupatoriochromene is therefore retardation of rate of germination of seeds, not inhibition. The amount of retardation caused by compounds **1** or **2** is dependent upon seed species and amount of time elapsed.

Seedling Growth Bioassay. Preliminary testing of seedling growth was done on lettuce using procedure A. In these tests, EUP slightly increases radicle and

TABLE 1. SEED GERMINATION

Seedling	Compound	Concentration	
		(ppm)	% of control
Lettuce	1	0	100.0
		10	98.9
		50	101.1
		100	82.1
	2	0	100
		10	71.6
		50	35.8
		100	46.3
Barnyard grass	1	0	100
		50	100
		100	98.9
	2	0	100
		50	92.3
		100	83.5
Red millet	1	0	100
		50	84.4
		100	88.5
	2	0	100
		50	74.0
		100	43.8
Perennial ryegrass	1	0	100
		50	90.2
		100	64.7
	2	0	100
		50	64.7
		100	17.6

hypocotyl growth at low concentrations (1 and 5 ppm, Table 2) but reduces their growth at 20 ppm. This effect is similar to that previously shown by sesquiterpene lactones isolated from YST (Stevens and Merrill, 1985). EUP has limited solubility in aqueous medium; hence, it was tested in the presence of Tween 20, a surfactant. The inhibitory effect on lettuce growth is noticed at lower concentration (5 ppm vs. 20 ppm), consistent with the predicted greater solubility of **1** in water when Tween 20 is present. The effect of EUP thus is concentration dependent; inhibitory effect is observed at higher concentrations.

Germination experiments showed some retardation in the presence of com-

TABLE 2. LETTUCE GROWTH IN EUP^a

Conc (ppm)	Radicle		Hypocotyl	
	(-)	(+)	(-)	(+)
0.0	100.0a	100.0a	100.0a	100.0a
1.0	108.8b	100.0a	100.0a	99.4a
5.0	102.2ab	72.1	104.0a	61.2
10.0	96.6a	44.0	100.2a	41.5
20.0	93.6	29.0	93.3	28.0
40.0	85.2	19.9b	76.5	23.2b
60.0	78.1	17.7b	68.5	20.7b
80.0	69.5c	17.7b	58.7b	21.9b
100.0	73.2c		60.8b	
200.0	49.6		35.9	

^a(-) Without Tween 20; (+) With Tween 20; values with the same letter in a vertical column are not significantly different ($\alpha = 0.05$) using a one-tailed Student *t* test and Mann-Whitney analyses.

pounds **1** and **2**. Thus, to measure the effect of these compounds on seedling growth alone, further bioassays were performed using pregerminated seedlings of comparable length (procedure B). The effect of EUP on the growth of other weeds and crop plants is shown in Figures 2–6. The radicle growth of barnyard grass is reduced at 1 ppm; cucumber and green foxtail at 5 ppm, red millet at 10 ppm, annual morning glory at 20, and alfalfa at 80 ppm. The hypocotyl growth of cucumber and barnyard grass is retarded at 1 ppm; red millet and green foxtail at 5 ppm, soybean and alfalfa at 10 ppm, and annual morning glory at 40 ppm. The inhibitory effects on root and hypocotyl growth by eupatoriochromene above compare favorably with those exhibited by nordihydro-guaiaretic acid, an allelochemical from creosote bush (Elakovich and Stevens, 1985); radicle growth of alfalfa, barnyard grass, lettuce, green foxtail, and red millet is inhibited at 10 ppm, and hypocotyl growth of lettuce at 5 ppm and green foxtail at 80 ppm. The studies here thus show that EUP inhibits both radicle and hypocotyl growth of test plants, although not necessarily at the same rate.

Encocalin (Figure 7) seems to reduce radicle and hypocotyl growth of lettuce seedlings gradually, with the reduction being significant at 60 ppm. After factoring in its effect on germination by using pregerminated seedlings in the bioassay (Figures 7–9), ENC still retards radicle and hypocotyl growth, but at higher concentrations than EUP. For example, radicle growth of barnyard grass is reduced at 10 ppm (*vs.* 1 ppm in **1**) and red millet at 40 ppm (*vs.* 10 ppm in **1**). The effect of the methylation of the 7-hydroxyl group of chromene **1** to **2** thus is to lower the phytotoxicity of **1** towards seedling growth.

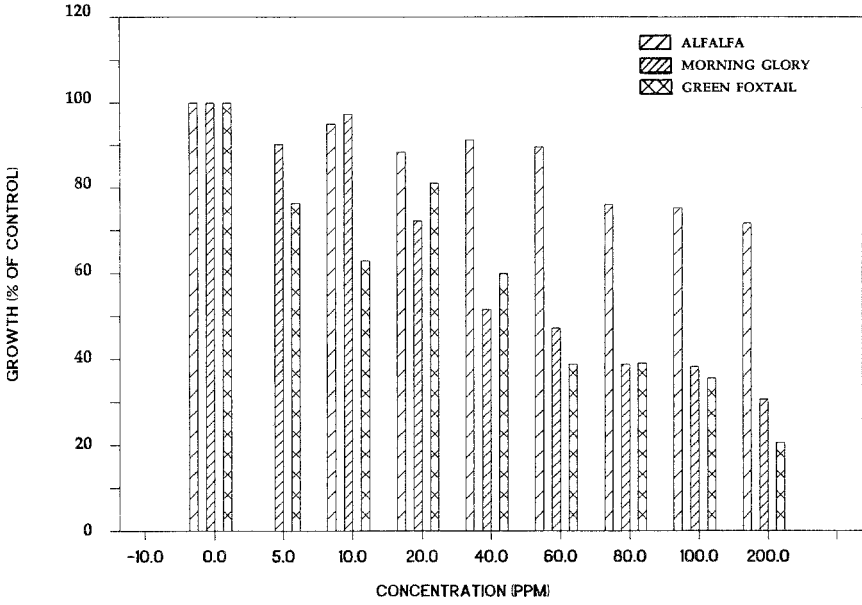


FIG. 2. Radicle growth in eupatoriochrome.

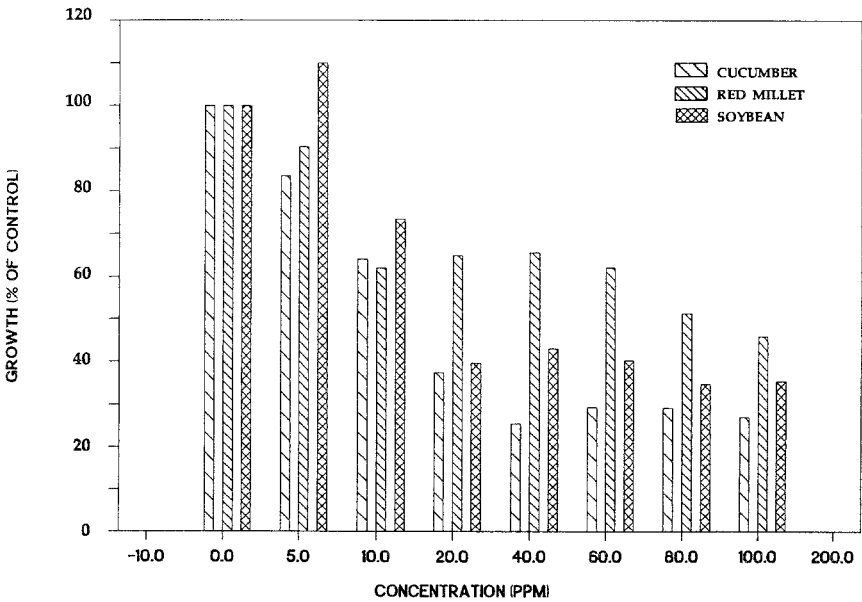


FIG. 3. Radicle growth in eupatoriochrome.

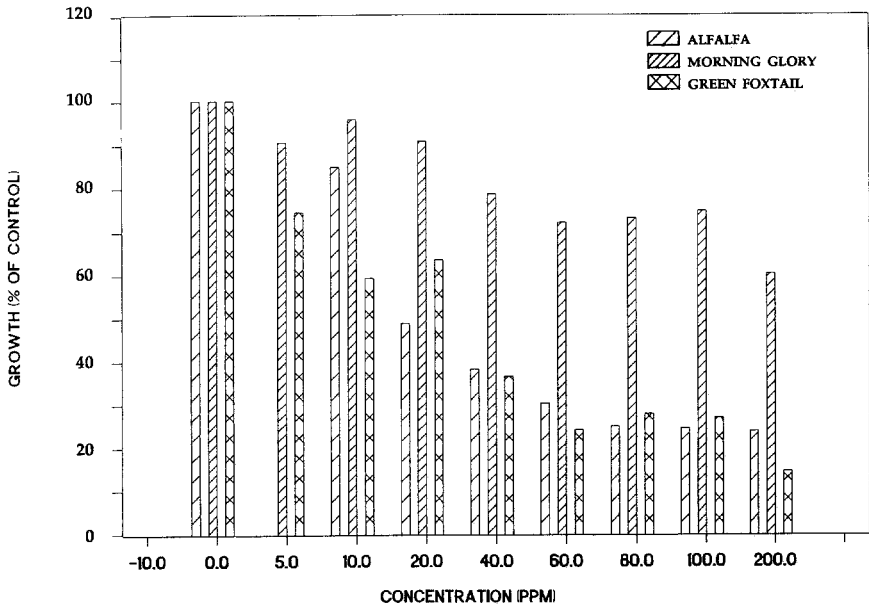


FIG. 4. Hypocotyl growth in eupatoriochromene.

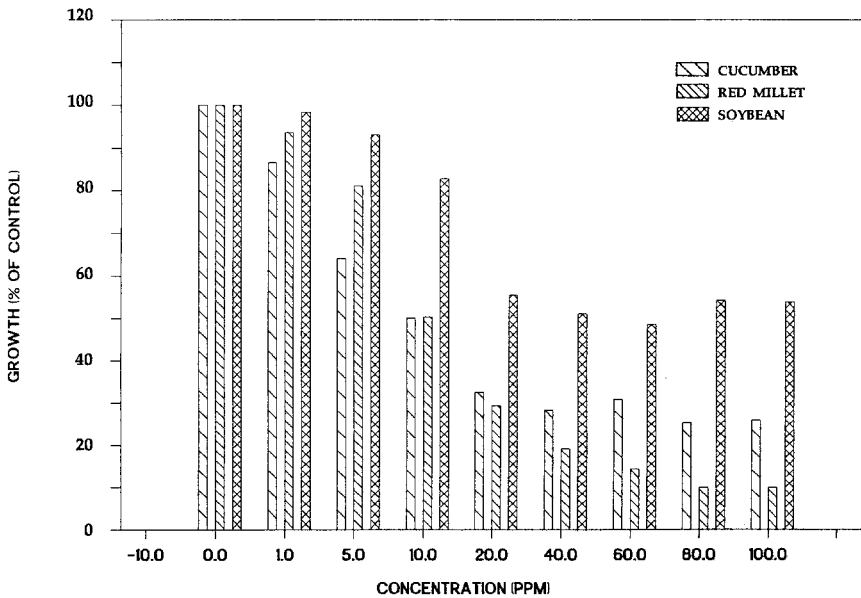


FIG. 5. Hypocotyl growth in eupatoriochromene.

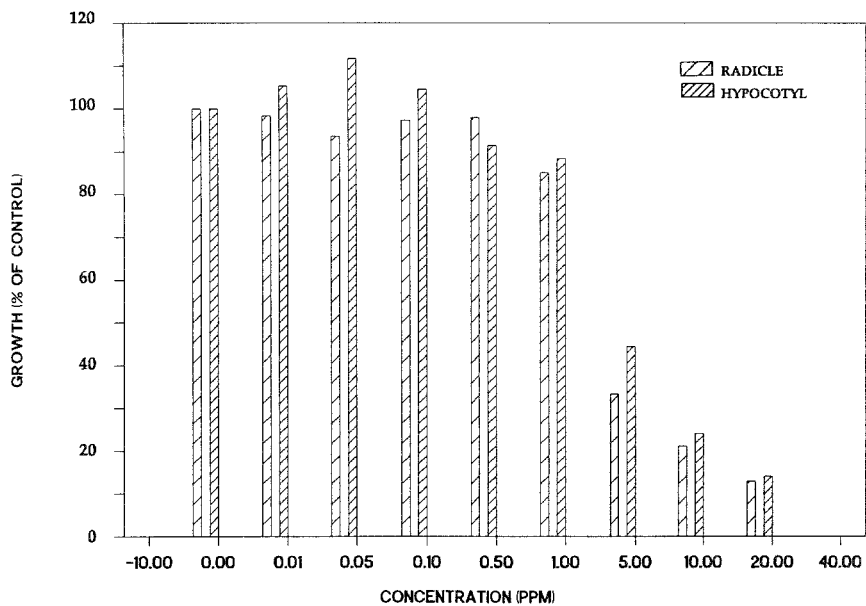


FIG. 6. Barnyard grass in eupatoriochrome.

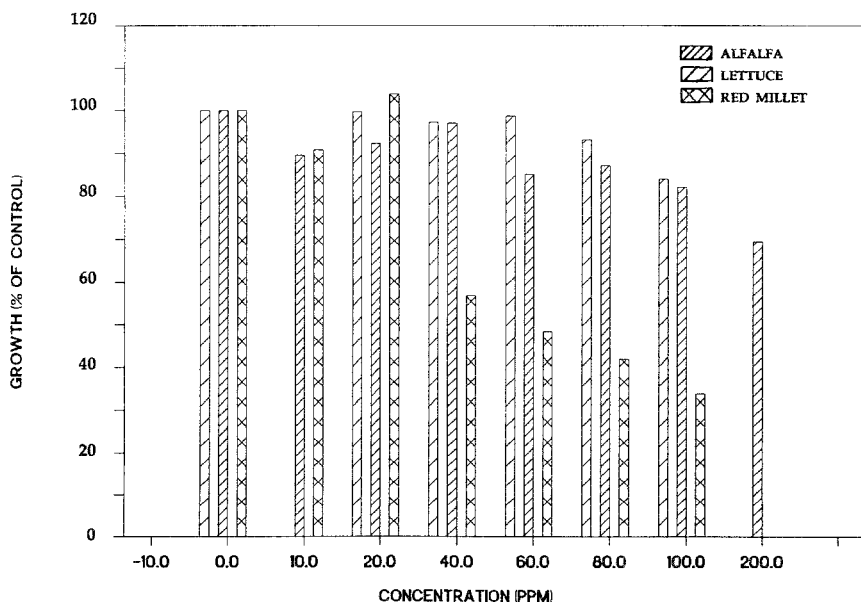


FIG. 7. Radicle growth in enecalalin.

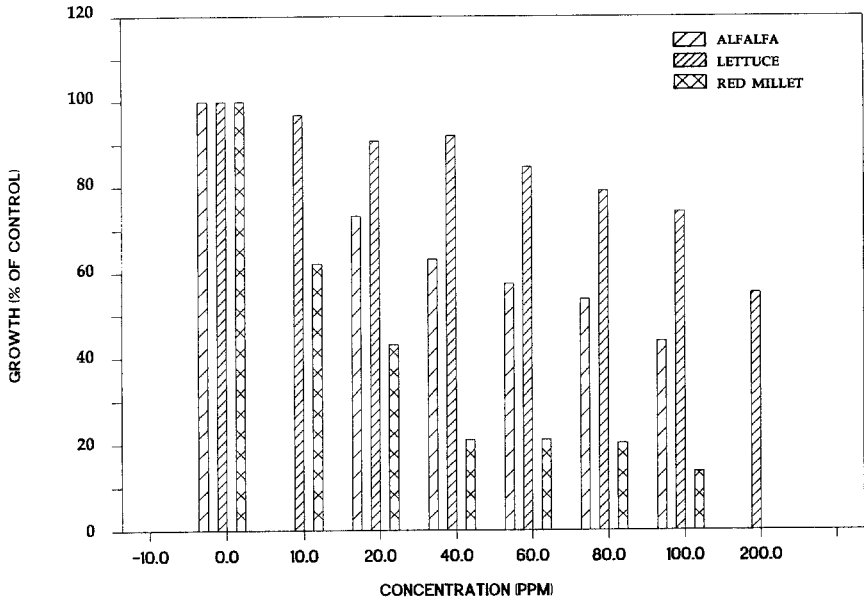


FIG. 8. Hypocotyl growth in enecalin.

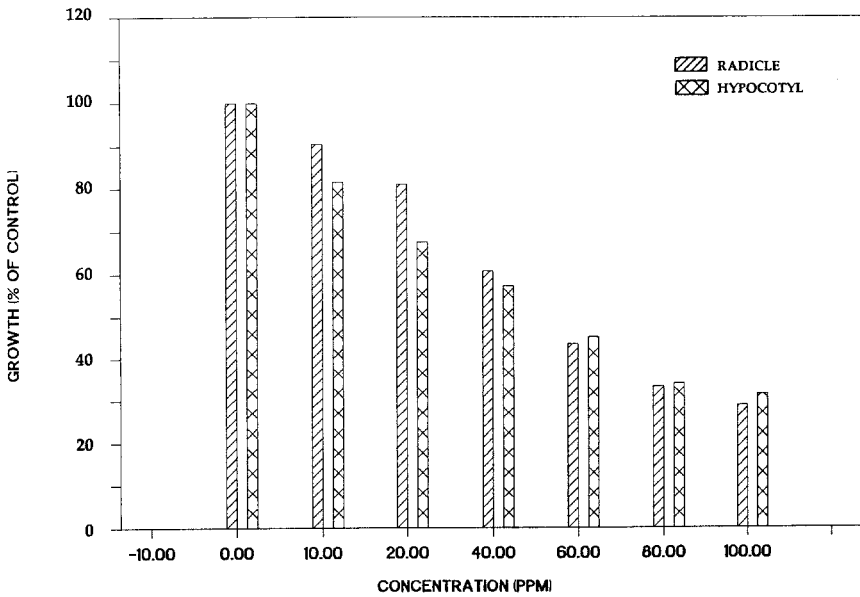


FIG. 9. Barnyard grass in enecalin.

Chromene **3** and chroman **5** were also tested to see what effect structural changes in the molecule has on biological activity. Again lettuce was used for preliminary testing. Red millet was also used because it was consistently one of the more sensitive seedlings among those previously tested. In these tests, both chromene **3** and chroman **5** (Figures 10 and 11) reduced hypocotyl growth as in tests with chromenes **1** and **2**, but at higher concentrations. However, chromene **3** and chroman **5** increase radicle growth for all concentrations tested. For example, at 80 ppm, radicle growth of red millet is 166% and 131% from compounds **3** and **5**, respectively. This is the reverse of the growth of retardation seen for EUP and ENC. The 7-hydroxyl (or 7-methoxyl) group and the double bond at the 3-position of EUP (or ENC) appear to be necessary for both hypocotyl and radicle growth retardation.

Root Formation. The enhancement of growth of some seedlings at low concentrations and the inhibition at higher concentrations seen in EUP is a characteristic growth pattern for auxins. Auxins are known to increase adventitious root formation. EUP was thus tested for this effect.

Mung Bean Cutting. EUP increases adventitious root formation of mung bean cuttings (Table 3). EUP increases the number of roots formed to over 200% of control at 20 ppm and above. When 1% acetone is used with the chromene, root promotion is observed at even lower concentrations (10 ppm).

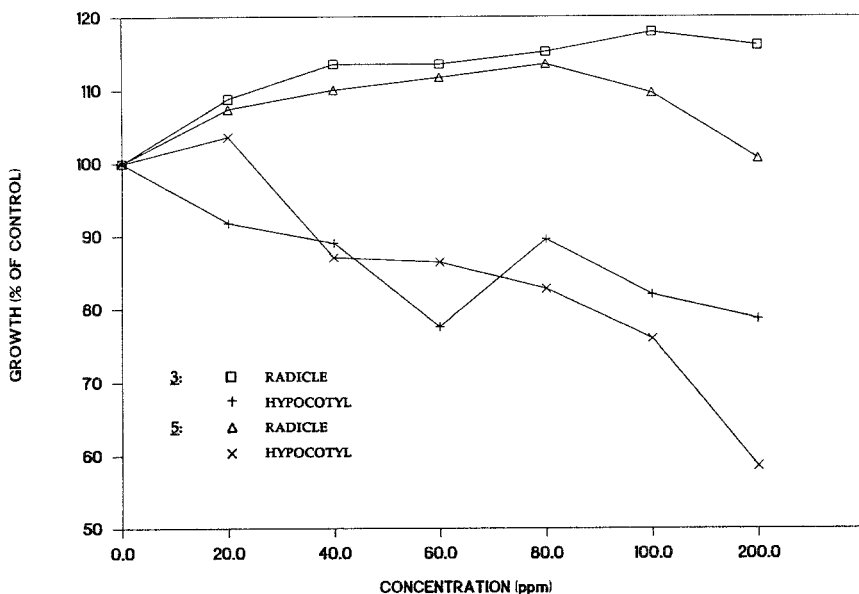


FIG. 10. Lettuce in **3** and **5**.

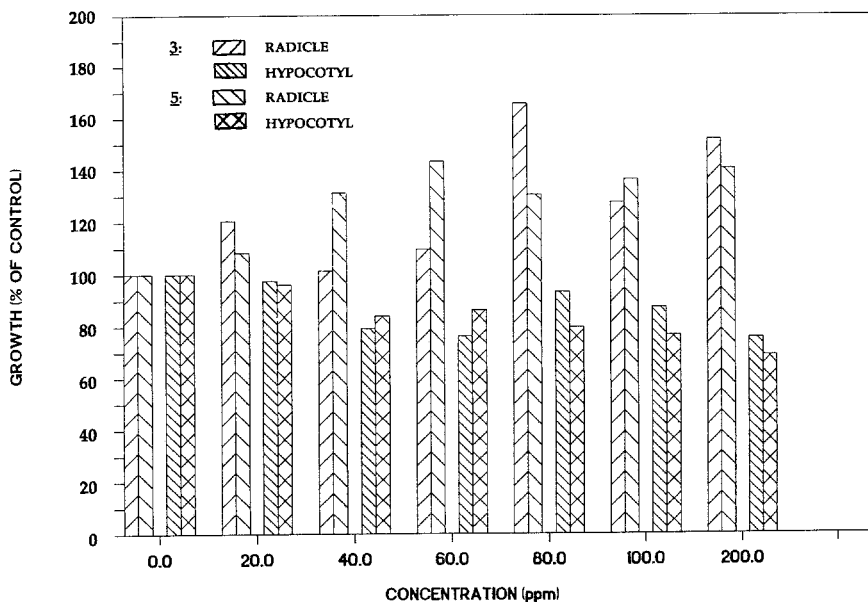


FIG. 11. Red millet in 3 and 5.

As was noted previously, EUP is nonpolar and is very insoluble in water. Presumably, acetone improves solubility of the compound into water, making it more readily available to the cutting at lower concentrations, thus its effect is seen at lower concentrations. The root-promoting activity of EUP (287%, 20 ppm) is comparable to those of indoleacetic acid ($\approx 300\%$, 18 ppm; Goodwin, 1978) and dehydrocostus lactone ($\approx 340\%$, 25 ppm; Kalsi et al., 1981).

Another test for auxin activity is the lanolin paste method. A lanolin paste of an auxin applied to the side of young mung bean hypocotyl seedlings enhances growth and produces a pronounced curvature (Goodwin, 1978). Such a curvature was not observed upon application of 1 to mung bean hypocotyls. Thus, eupatoriochromene is not an auxin, but rather a plant growth regulator.

Effect of Eupatoriochromene and Encecalin on Yellow Starthistle (YST). The effect of EUP and ENC on YST is shown in Figure 12. Although germination is virtually unaffected by either EUP or ENC, both radicle and hypocotyl growth of seedlings are retarded by the two chromenes. In fact, YST is one of the most sensitive seedlings among those tested. Eupatoriochromene and encecalin affect its growth at 1 ppm and 10 ppm, respectively. Growth retardation increases with time.

When seedlings in the germination experiments were allowed to grow in the dark for several days, marked differences were noted in growth and mor-

TABLE 3. ADVENTITIOUS ROOT-FORMATION OF MUNG BEAN SEEDLINGS IN EUPATORIOCHROMENE

Conc (ppm)	With acetone		Without acetone	
	No. of roots	% of control	No. of roots	% of control
0	9.3	100	9.3	100
10	21	226	9.3	100
20	26.7	287	20	210
40	22.7	244	20.3	216
80	21.7	233	25.7	272

tality. The radicles and hypocotyls of seedlings in the control agar solution continued to elongate. There was one root per seedling. After a few days in the agar solutions containing test compounds, hypocotyl and radicle growth seemed to cease; the hypocotyl was short and fat; the radicle began to send out lateral roots (one to three per plant). These findings are consistent with previous experiments. For two weeks all seedlings lived. At 2½ weeks, the roots of seedlings in test solutions (50 ppm and 100 ppm) appeared to have darkened and some

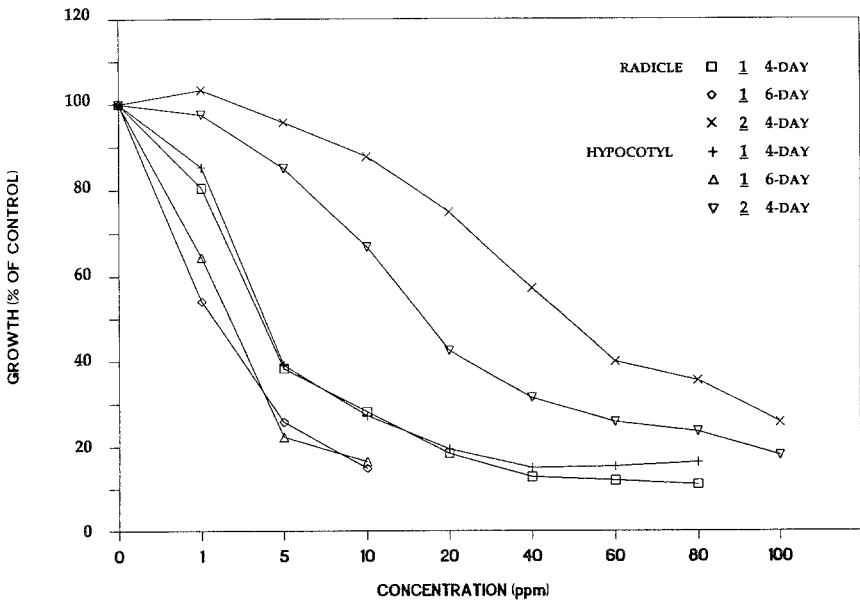


FIG. 12. Yellow starthistle in 1 and 2.

seedlings were dying. At three weeks, all seedlings in the control and test solutions were dead. These experiments showed that the growth of YST is retarded in the presence of EUP and ENC, but that the plant can survive for an extended period in their presence.

The effects of eupatoriochromene (1) and enecalinalin (2) on the regulation of plant growth is the first report of chromenes having this type of activity. These compounds, together with the sesquiterpene lactones also found in YST and shown to possess plant growth activity (Stevens and Merrill, 1985), may play a key role in the allelopathic nature of these noxious weeds.

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ELECTROANTENNOGRAMS BY MOUNTAIN PINE BEETLES, *Dendroctonus ponderosae* HOPKINS, EXPOSED TO SELECTED CHIRAL SEMIOCHEMICALS

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Abstract—Electroantennograms (EAGs) were recorded from *D. ponderosae* to the enantiomers of the terpenoid bark-beetle pheromones *trans*-verbenol, *cis*-verbenol, verbenone, and the bicyclic ketals frontalin, *exo*-brevicommin, and *endo*-brevicommin. Male and female responses to enantiomers of the terpenoids differed significantly only at the two highest concentrations. No sex differences were seen in response to the bicyclic ketals. Significantly different responses to the enantiomers of all the chemicals, except frontalin, were noted over at least part of the dosage–response ranges tested. The negative antipode for all of the terpenoids elicited higher responses, while for the bicyclic ketals, the positive antipode effected the largest responses except for the two highest concentrations of *exo*-brevicommin.

Key Words—Olfaction, chirality, pheromone, semiochemical, enantiomer, bark beetle, electrophysiology, electroantennogram, mountain pine beetle, *Dendroctonus ponderosae*, Coleoptera, Scolytidae.

INTRODUCTION

Several species of pine trees in western North America are susceptible to the highly destructive attack of the mountain pine beetle (MPB) *Dendroctonus ponderosae* Hopkins. Colonization of the host tree depends on the beetles' responses to a complex blend of aggregation pheromones and host-tree kairomones (Vité and Gara, 1962; McCambridge, 1967; Pitman and Vité, 1969). *trans*-Verbenol, identified as the main female-produced pheromone (Pitman et al., 1968), is ineffective by itself (Pitman and Vité, 1969; McKnight, 1979), but when combined with the host-tree terpenes α -pinene or, especially, myrcene (Merrifield, 1972; Billings, 1974; Billings et al., 1976; Pitman et al., 1978), it becomes an effective aggregation pheromone. The addition of small quantities of *exo*-brevicommin to *trans*-verbenol plus myrcene (Borden et al., 1983a,b; Conn et al., 1983) produces a highly effective blend capable of attracting both sexes and mediating mass attack on several hard-pine species.

Investigations of chiral sensitivity in bark beetles began with *Scolytus multistriatus* when its aggregation pheromone was found to contain a three-component blend of (-)-enantiomers (Lanier et al., 1976). Since then, chiral discrimination has been shown in a number of other species of bark beetles, as well as in many other insects (Silverstein, 1979).

Research on optical isomers of pheromones of the mountain pine beetle began when significant differences were found in the behavior of the MPB to several semiochemicals, especially *trans*-verbenol (McKnight, 1979). Racemic verbenone and (-)-verbenone, but not (+)-verbenone, were found to have antiaggregative properties in both field and lab tests of beetles associated with lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann (Ryker and Yandell, 1983). However, both enantiomers were active when tested on beetles from ponderosa pine, *Pinus ponderosa* Dougl. ex Laws. Male MPB from Oregon and British Columbia were found to produce predominantly (+)-*exo*-brevicommin and (+)-*endo*-brevicommin (Schurig et al., 1983). MPB in white pine in Idaho and lodgepole pine in Oregon responded best to (-)-*trans*-verbenol (Libbey et al., 1985).

Electroantennograms to racemic semiochemicals were recorded from MPB (Whitehead, 1986). The purpose of this paper is to report results of an investigation of antennal olfactory responses of the MPB to specific chiral pheromones, most of which have been determined to be important in the behavior of the insect.

METHODS AND MATERIALS

Adult beetles were obtained from infested bolts of lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, cut in Logan Canyon, Utah. After emergence, the beetles were sexed (Lyon, 1958) and placed on moist strips of filter

paper in Petri dishes at 5°C until use within 30 days after emergence. No differences in response were detected from beetles of different ages.

Electroantennogram (EAG) techniques were described previously (Whitehead, 1986) and were modified from those reported by Dickens et al. (1983). Briefly, EAGs were recorded with Ag-AgCl capillary electrodes filled with insect saline (Yamasaki and Narahashi, 1959). The recording electrode was placed into the distal end of the antennal club following prepuncture with an electrolytically sharpened tungsten needle. The indifferent electrode was placed into the head via the insect's mouth. EAG signals were amplified by a Grass P-16 amplifier and plotted on graph paper by an Apple II microcomputer. Chemicals were diluted in *n*-pentane in decade steps. A 10- μ l sample was placed on a 20- \times 6-mm filter-paper strip and inserted into an 80- \times 5-mm glass cartridge. The stimulus was made by orienting the cartridge toward the preparation, the tip being about 1 cm away, and by passing a 1-sec flow of air through the cartridge at 1.7 liter/min. Samples were presented from lowest to highest concentrations. Five replicates (different beetles) for each sex were made at each concentration. At least 4 min were allowed between stimuli, except at higher concentrations, when 5 min were allowed (Dickens et al., 1983; Whitehead, 1986). Racemic frontalin (10 μ g on filter paper) was used as a standard and was applied between every set of two stimulations of enantiomeric chemicals. EAGs were measured for negative amplitude and were shown as a percent of the mean of the two bracketing standards.

The semiochemicals used in this study are listed in Table 1. The optical purities of *trans*- and *cis*-verbenol and verbenone were determined using a chiral NEB column fitted gas chromatograph (Bradshaw et al., 1987). Optical purities of *exo*- and *endo*-brevicommin and frontalin were published by Mori (1974, 1975, 1985) and confirmed by chiral gas chromatography. The chemical purities of all the compounds were determined by gas chromatography. The temperature program initial value was set at 40°C for 2 min, increasing at a rate of 4°C/min until 150°C final temperature was reached (Whitehead, 1986).

Response to 10 μ l of *n*-pentane on filter paper was measured but not subtracted from the responses to the other chemicals. Responses to air were subtracted from the test-chemical reaction. We did a repeated-measures ANOVA (Myers, 1979) to control for the differences in measuring between beetles and across the same beetle.

RESULTS

EAGs obtained in response to the test chemicals were similar to those recorded previously from the MPB (Whitehead, 1986) and those from other species of beetles (Angst and Lanier, 1979; Grant and Lanier, 1982; Dickens, 1981; Dickens, et al., 1983). Increases in stimulus concentrations yielded increases in response amplitude (Figures 1-3). Saturation occurred at 100 μ g

TABLE 1. SOURCES AND PURITIES OF CHEMICALS

Compound	Chemical purity (%)	Optical purity (%)	Source ^a
Bicyclic ketals			
(+)- <i>endo</i> -Brevicommin	100	100	A
(-)- <i>endo</i> -Brevicommin	100	100	A
(+)- <i>exo</i> -Brevicommin	100	97.1	A
(-)- <i>exo</i> -Brevicommin	100	100	A
(+)-Frontalin	100	>98	A
(-)-Frontalin	100	>98	A
(±)-Frontalin ^b	99.6	—	C
Terpenoids			
(+)- <i>cis</i> -Verbenol	98.4	93.5	B
(-)- <i>cis</i> -Verbenol	99.1	95.4	B
(+)- <i>trans</i> -Verbenol	94.7	85.8	B
(-)- <i>trans</i> -Verbenol	93.5	91.6	B
(±)-Verbenone	93.8	92.9	B
(-)-Verbenone	91.8	95.5	B

^a A = K. Mori; B = H. Wieser and E.A. Dixon, Department of Chemistry, University of Calgary, Alberta, Canada; C = Albany International, Willoughby, Ohio.

^b Used for standard.

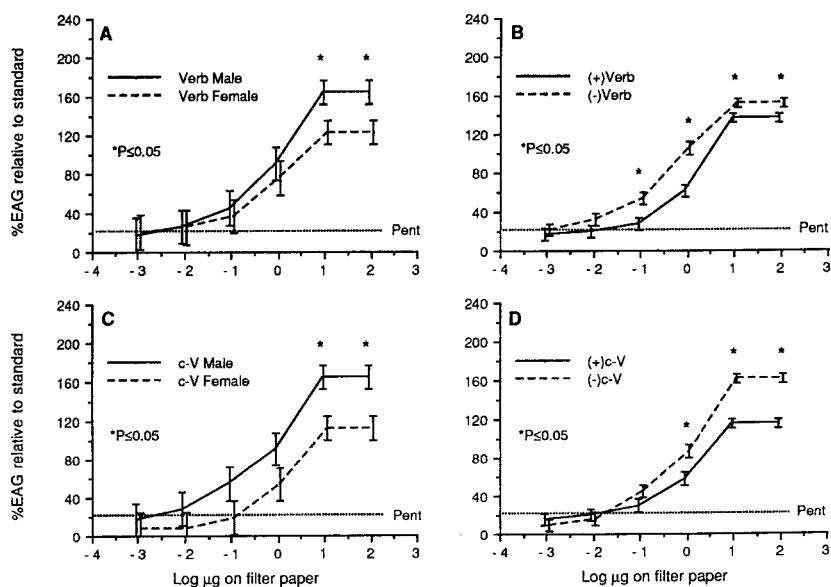


FIG. 1. (A,C) EAG-derived curves of MPB males (solid lines) and females (dashed lines) to serial dilutions of verbenone and *cis*-verbenol (enantiomeric responses averaged) and EAG-derived curves (responses from sexes averaged) to serial dilutions of (+)-enantiomer (solid lines) and (-)-enantiomer (dashed lines) of verbenone (B) and *cis*-verbenol (D). $N = 10$; error bars indicate \pm SEM. *Significant difference between responses at 0.05 level. Verb = verbenone, c-V = *cis*-verbenol. Horizontal dotted line = pentane response.

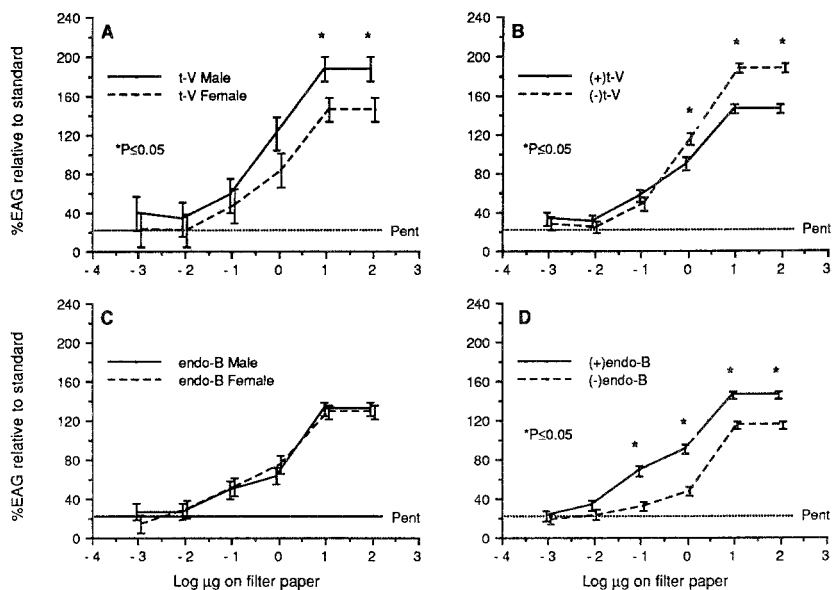


FIG. 2. (A,C) EAG-derived curves of MPB males (solid lines) and females (dashed lines) to serial dilutions of *trans*-verbenol and *endo*-brevicomin (enantiomeric responses averaged) and EAG-derived curves (responses from sexes averaged) to serial dilutions of (+)-enantiomer (solid lines) and (-)-enantiomer (dashed lines) of *trans*-verbenol (B) and *endo*-brevicomin (D). $N = 10$; error bars indicate \pm SEM. *Significant difference between responses at 0.05 level. t-V = *trans*-verbenol, endo-B = *endo*-brevicomin. Horizontal dotted line = pentane response.

for all chemicals except frontalin where, because of the limited amount of product on hand, the highest concentration tested was $10 \mu\text{g}$. The pentane control produced responses of $21.8 \pm 15.14\%$ (SE) of the frontalin standards (Figures 1–3). There were no significant differences between the male and female responses. The frontalin standard yielded 0.48 ± 0.01 mV (SE) from males and 0.49 ± 0.01 (SE) mV responses from females, which were also nonsignificant. Few responses to air were noted.

The analysis of the terpenoids showed that the males and females responded differently at each concentration level (Figures 1A,C and 2A). As the concentration of the terpenoids increased, there was usually a greater separation between the points on the curves. The analysis of the bicyclic ketals showed no differences by sex (Figures 2C and 3A,C) but did show a chemical by enantiomer interaction not found in the terpenoids.

Figures 1–3 indicate that the magnitude of the responses were dependent upon the chemical, its concentration, and its enantiomer. There were statistically different responses between the sexes at the two highest concentrations of all the terpenoids (Figures 1A,C and 2A). In all cases where sex differences were noted, the males showed the largest responses.

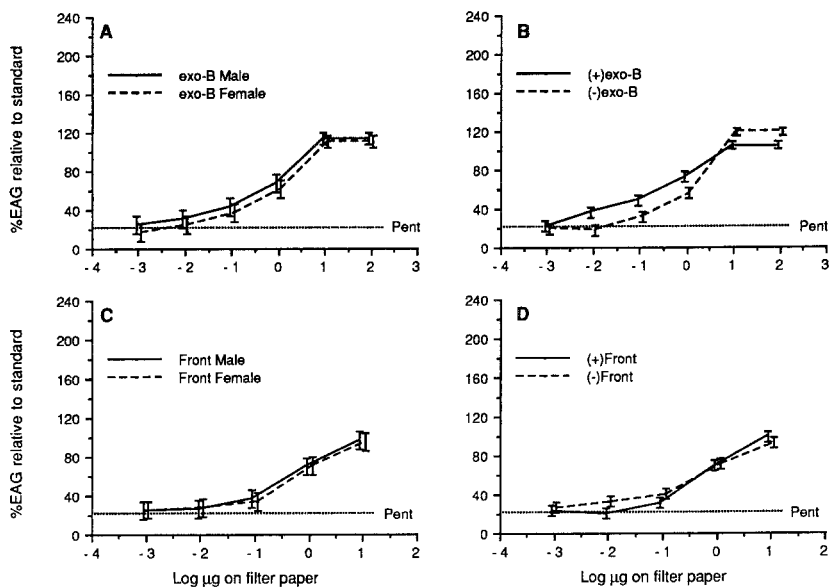


FIG. 3. (A,C) EAG-derived curves of MPB males (solid lines) and females (dashed lines) to serial dilutions of *exo*-brevicomin and frontalin (enantiomeric responses averaged) and EAG-derived curves (responses from sexes averaged) to serial dilutions of (+)-enantiomer (solid lines) and (-)-enantiomer (dashed lines) of *exo*-brevicomin (B) and frontalin (D). $N = 10$; error bars indicate \pm SEM. *exo*-B = *exo*-brevicomin, Front = frontalin. Horizontal dotted line = pentane response.

Figure 4A,B shows the values of the response averaged across concentration for sex, chemicals, and chirality. Figure 4A shows that frontalin was not significantly different from *exo*-brevicomin; however, both compounds were different from *endo*-brevicomin. The responses to the bicyclic-ketal enantiomers were statistically different from each other. Figure 4B, on the other hand, shows that both the sex and chirality factors for the terpenoids were different but that the overall responses to verbenone and *cis*-verbenol were not different from each other, while the response to *trans*-verbenol was different from those to verbenone and *cis*-verbenol when averaged across concentration. However, because of the interactions, these results could change for a given concentration level, e.g., in Figure 2B the responses to enantiomers were the same for the lowest concentrations but different for the highest concentrations.

Responses to the enantiomers of the terpenoids and the bicyclic ketals were more pronounced than the influence of the sex of the beetle. However, the analysis showed that no significant differences existed between responses to the frontalin enantiomers (Figure 3D). On the other hand, (+)-*exo*-brevicomin (Figure 3B), (+)-*endo*-brevicomin (Figure 2D) and (-)-verbenone (Figure 1B)

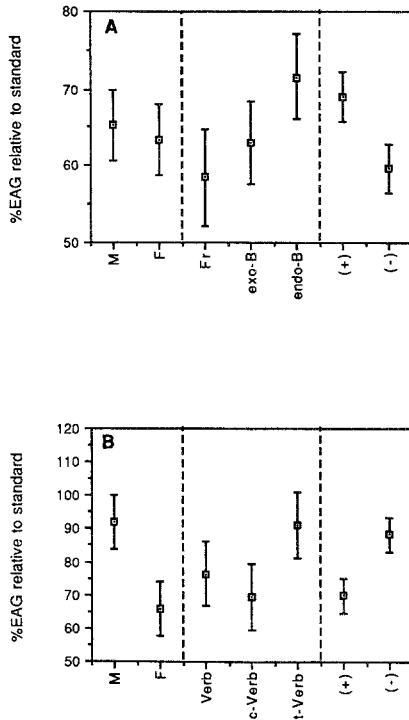


FIG. 4. Estimated means of the EAG responses by sex, chemicals, and enantiomers averaged over concentration. M = male, F = female, Fr = frontalinal, exo-B = *exo*-brevicommin, endo-B = *endo*-brevicommin, Verb = verbenone, c-Verb = *cis*-verbenol, t-Verb = *trans*-verbenol, (+) = (+)-enantiomer, (-) = (-)-enantiomer. $N = 180$ (sex), 120 (chemical), 180 (enantiomers). Vertical dashed lines indicate that the enclosed averaged responses should not be compared to the responses within the other dashed lines.

had the largest responses over most of their concentration ranges. (-)-*cis*-Verbenol (Figure 1D) and (-)-*trans*-verbenol (Figure 2B) showed significantly greater responses than their antipodes at the three highest concentrations. In general, responses to (-)-enantiomers were greater for the terpenes; while, by comparison, responses to (+)-enantiomers were greatest for the bicyclic ketals.

DISCUSSION

Our results confirm some of the findings of previous workers investigating the effects of chiral semiochemicals on the MPB both in laboratory and in field studies. McKnight (1979) found that the MPB responded significantly better to

(-)-*trans*-verbenol than to the (+)-antipode in white pine stands in Idaho and lodgepole pine stands in Oregon. This was supported by Libbey et al. (1985), who determined that males responded better to the (-)-antipode while females were attracted equally. Borden et al. (1987) indicated that both (\pm)- and (-)-*trans*-verbenol caused significant attraction of both sexes, while the (+)-enantiomer did not, and they determined by gas chromatographic analysis that females from British Columbia contained 65–87% of (-)-*trans*-verbenol.

Ryker and Yandell (1983) determined in field trapping that both sexes of the MPB showed significantly reduced attraction to the attractive mixture of *trans*-verbenol, myrcene, and α -pinene when exposed to either (-)- or (\pm)-verbenone in both ponderosa and lodgepole pine stands. However, their laboratory bioassay demonstrated that (\pm)-verbenone was effective in reducing the arrestment of male MPB and the number emitting attracting chirps, while either enantiomer would reduce arrestment. On the other hand, only the (-)-antipode was effective in reducing arresting chirps. They concluded that the antiaggregative effect of (-)-verbenone is not changed by the presence of its antipode in the racemic mixture. Verbenone is now recognized as an inhibitor of aggregation behavior in several bark beetle species (Borden, 1982). Borden et al. (1987) confirmed that verbenone was inhibitory to MPB males at several release rates in field studies.

The role of *cis*-verbenol is unclear in the biology of the MPB. Pitman et al. (1969) failed to find major quantities in either sex; however, it has been identified in female frass (Ryker and Rudinsky, 1982; Pierce et al., 1987). It was significantly more effective in eliciting EAG responses in male MPB than was *trans*-verbenol but not in females (Whitehead, 1986); however, in our current study, the opposite occurred, wherein males were slightly more sensitive to *trans*-verbenol than to *cis*-verbenol. The reasons for this disparity are unclear. Possibly the relative impurity of compounds (90%) used in the previous study (Whitehead, 1986), the low numbers of test animals ($N = 5$), or errors in recording in both studies could be contributing factors. In *Dendroctonus pseudotsugae*, *cis*-verbenol stimulates more generalized synergist cells (Dickens et al., 1984). The MPB may have similar cells, and if *cis*-verbenol fills the receptor sites on these cells in a similar fashion to *trans*-verbenol, then more or less equal responses may be expected from both compounds.

exo-Brevicomin is a female attractant at low concentrations (Conn et al., 1983; Rudinsky et al., 1974), but high release rates resulted in inhibition of male responses while intermediate doses yielded partial inhibition (Borden et al., 1987), thus suggesting that *exo*-brevicomin may be a multifunctional pheromone (Rudinsky et al., 1974). Analysis of male MPBs from Oregon and British Columbia showed that they produce predominantly (+)-*exo*-brevicomin and (+)-*endo*-brevicomin (Schurig et al., 1983). Their findings correlate positively with our observations that MPB antennal sensilla are more sensitive to (+)-*exo*-brevicomin than its antipode. However, field tests showed that the beetles seem

to be insensitive to chirality, in that they responded to racemic *exo*-brevicomin in a similar fashion as to the enantiomers (Borden et al., 1987).

The role of *endo*-brevicomin in the biology of the beetle is also unclear. It has been reported as a male-produced pheromone that acts as an antiaggregant similar to *exo*-brevicomin (Rudinsky et al., 1974). Ryker and Rudinsky (1982) suggested that high levels of both racemic *exo*- and *endo*-brevicomin may act as antiaggregative pheromones in lodgepole pine infestations. However, Borden et al. (1987) report that their unpublished experiments have failed to confirm any attractive or antiaggregative properties for *endo*-brevicomin in lodgepole pine forests. Our results showed that the (+)-enantiomer elicited significantly higher EAGs than its antipode and the enantiomers of *exo*-brevicomin. No other known field or laboratory studies have been carried out to date on the effects of *endo*-brevicomin enantiomers.

At low release rates, frontalin has been implicated recently as an aggregation pheromone in females, and at high release rates as an antiaggregative pheromone; furthermore, neither enantiomer was attractive at low rates, but they were inhibitory at high rates compared to the racemate (Borden et al., 1987), suggesting an additive effect of both enantiomers (Dickens et al., 1985). However, our results suggest that the beetle is incapable of detecting differences between the enantiomers at any concentration, and also, contrasting with the field data, both sexes had about the same sensitivity to frontalin (Whitehead, 1986) (Figure 3C).

A new series of experiments, being undertaken using extracellular recordings from individual antennal sensilla, may clarify some of the unanswered questions regarding semiochemical detection by the MPB.

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CHARACTERIZATION AND ECOLOGICAL
IMPLICATIONS OF MIDGUT
PROTEOLYTIC ACTIVITY
IN LARVAL *Pieris rapae*
and *Trichoplusia ni*

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Abstract—In their larval luminal midgut fluid, *Trichoplusia ni* (Lepidoptera: Noctuidae) and *Pieris rapae* (Lepidoptera: Pieridae) contain endopeptidases as their primary proteases. Neither species has detectable exopeptidase activity. Studies using enzyme-specific substrates and inhibitors demonstrate that the endopeptidases are serine proteinases (both trypsinlike and chymotrypsinlike) with histidine at the active site. Optimal pH for the tryptic and chymotryptic activity is 8.5 and 8.0, respectively, for *T. ni*. and 8.0 and 9.0, respectively, for *P. rapae*. The efficiency of proteolytic digestion (as measured by the rate of in vitro digestion of a standard protein by the midgut luminal fluid) is positively correlated with the larval dietary protein requirement and is significantly influenced by the ratios of tryptic to chymotryptic activity present in the gut lumen of these two species of Lepidoptera.

Key Words—*Pieris rapae*, *Trichoplusia ni*, Lepidoptera, Pieridae, Noctuidae, proteases, digestive enzymes, proteinase inhibitors, dietary protein requirement.

INTRODUCTION

One determinant of the success of herbivorous insects on specific host plant(s) is the ability to acquire essential nutrients in appropriate amounts. The digestive system in herbivores has adapted to specific food sources by (1) synthesizing

specific classes of digestive enzymes (Baker, 1981), (2) regulating the level of secretion of those enzymes (Broadway and Duffey, 1986a; Baker, 1977), and (3) maintaining the pH of the gut lumen within a relatively narrow range (Berenbaum, 1980) for optimal enzyme activity. For example, the cowpea-eating bruchid beetle has an acidic gut that secretes a thiol enzyme as its primary protease (Gatehouse et al., 1985), whereas the detritus-feeding larval caddisfly has a mildly basic midgut containing extremely high proteolytic activity (Martin et al., 1981), and larval *Heliothis zea*, a generalist herbivore, has a strongly alkaline midgut with relatively low proteolytic activity contributed primarily by a serine (trypsinlike) protease (Broadway and Duffey, 1986a). The general condition of the gut lumen (i.e., pH, ionic strength), and the class(es) and quantity of the intestinal digestive protease(s) determine: (1) the quantity and quality of protein that can be digested and, therefore, the level of nutrients available to the insect (Broadway and Duffey, 1986a, 1988); (2) the ability of naturally occurring inhibitors (common in plants) to block proteolytic digestion (Hilder et al., 1987; Broadway et al., 1986; Gatehouse et al., 1985); and (3) the susceptibility of insects to pathogens (Espinoza-Fuentes and Terra, 1987; Cha-Yun and Chen, 1982; Narayanan et al., 1976) and plant natural products (Mole and Waterman, 1987; Martin and Martin, 1984; Meisner et al., 1978; Goldstein and Swain, 1965). Thus, characterization of the midgut luminal milieu of herbivorous insects is necessary to gain insight into the acquisition of essential nutrients and to determine the physiological effects and limitations of phytochemicals that may modify digestion (e.g., phenolics, saponins, and proteinase inhibitors).

One group of phytochemicals that can significantly influence insect digestive physiology is the proteinase inhibitors. Plant proteinase inhibitors can effectively reduce proteolytic activity *in vitro* and significantly inhibit the growth of specific herbivorous insects (Hilder et al., 1987; Broadway and Duffey, 1986b). However, these inhibitors are only effective when susceptible digestive enzyme(s) and appropriate physiological conditions (i.e., pH, ionic strength) are present (Gatehouse et al., 1985).

Trichoplusia ni (Hübner), the cabbage looper, and *Pieris rapae* (L.), the imported cabbageworm, are major pests on a number of economically important agricultural crops. *T. ni* is a polyphagous herbivore, feeding on a broad spectrum of crops including cotton, cabbage, soybean, and tomato, whereas *P. rapae* is oligophagous, restricting its herbivory to plants in the family Cruciferae (Metcalf et al., 1962). All of these crops contain proteinase inhibitory activity, but the level of activity is highly variable (Broadway, 1988; Whitaker, 1981; Chen and Mitchell, 1973). Although there are extensive reports on behavior and host distribution of both of these species of Lepidoptera (Renwick and Radke, 1988; Maguire, 1984a,b; Gothilf and Beck, 1967; Shorey et al., 1962), no detailed information has been published on the digestive physiology of either species. This study presents information about the physiological condition and

protease profile of the larval midgut lumen of these two species of herbivorous Lepidoptera. The larval dietary protein requirement and the ability of specific proteinase inhibitors to reduce the activity of larval digestive enzymes *in vitro* are also described.

METHODS AND MATERIALS

Insects

All the insects used for this study were provided by Dr. A.M. Shelton, NYS Agricultural Experiment Station, Geneva, New York. The colony of *Trichoplusia ni* was established with individuals collected in the field in 1987. Larvae were reared to pupation on a pinto bean-based artificial diet (Shorey and Hale, 1965) in groups of 15–20 individuals per 8-oz wax-coated paper cup. The cups were placed in an environmental chamber maintained at 27°C, 70% relative humidity, with a photoperiod of 16:8 light–dark, until the larvae pupated. Groups of 60–100 pupae were placed in a $\frac{1}{4}$ -in. wire-mesh cylindrical cage (30 cm high \times 20 cm diameter), maintained in the environmental chamber. After eclosion, the adults were provided with 10% sugar water for food and waxed paper for oviposition. The waxed paper containing eggs was collected daily, divided into sections containing 15–20 eggs, and each section was stapled to the top of a diet cup. The neonates were provided with artificial diet *ad libitum*.

The laboratory colony of *Pieris rapae* was established from adults collected in the field in 1982 and was maintained as described by Webb and Shelton (1988). Larvae were reared to pupation on a high wheat germ-based artificial diet in groups of 30–40 individuals per 16-oz styrofoam cup. The cups were placed on their sides in an environmental chamber maintained at 21°C, 40% relative humidity, with a photoperiod of 16:8 light–dark, until the larvae pupated. Groups of 500 pupae were placed in a fine mesh cage (92 \times 70 \times 106 cm), in a greenhouse (25°C, 3200 lux light provided by a 2000-W metal halide lamp). The adults were provided with 10% sugar water containing 0.15% yellow food coloring for food and parafilm baited with a broccoli leaf for oviposition. The parafilm containing eggs was collected after a 1-hr oviposition period, surface-sterilized with 10% formalin, rinsed with sterile water, and air-dried. The parafilm was then cut into sections containing 50 eggs, and each section was placed in a cup containing artificial diet.

Isolation and Characterization of Proteolytic Enzymes

Proteolytic enzymes were isolated from the midgut lumen of fifth-instar larvae that were actively feeding on artificial diet to ensure optimal enzyme activity (Broadway and Duffey, 1986a). Whole midguts of 50–60 actively feeding fifth-instar larvae per species were quickly removed, wiped dry (to remove

external hemolymph), then placed in an ice-cold centrifuge tube. The midguts were gently homogenized to release all luminal fluid, then centrifuged at 20,000g, 4°C for 10 min, and the supernatant was analyzed for protease activity.

The survey of proteolytic activity in the midguts of fifth instar larval *P. rapae* and *T. ni* included assays for general protease activity, exopeptidase (carboxypeptidases A and B), and endopeptidase (chymotrypsin and trypsin) activity. Enzyme-specific substrates were used for activity determination of each enzyme.

General protease activity was measured using azocoll (Sigma Chemical Co., St. Louis, Missouri), a proteolytic substrate (Martin et al., 1981). A 50- μ l aliquot of insect enzyme (diluted 1:3 with 0.1 M phosphate buffer, pH 8) was mixed with 10 mg of azocoll in 167 μ l 0.1 M phosphate buffer, pH 8, and incubated at room temperature for exactly 15 min. The reaction was stopped by the addition of 1 ml 10 mM HCl. The solution was centrifuged for 30 sec in a microcentrifuge, then measured spectrophotometrically at 520 nm. Each test solution was replicated 10 times.

Exopeptidases. For the determination of carboxypeptidase A activity, 1 mM HPLA (hippuryl-L-phenylalanine, Sigma) in 0.05 M Tris buffer, pH 7.5, containing 0.5 M NaCl was used (McClure et al., 1964). A 100- μ l aliquot of supernatant from the gut homogenate (diluted 1:3 with buffer) was added to 2.9 ml substrate-buffer mixture, and activity was monitored spectrophotometrically at 254 nm for 3 min. Carboxypeptidase A from bovine pancreas (Sigma) was used as a standard. The assay was replicated three times.

Carboxypeptidase B activity was measured using 1 mM HA (hippuryl-L-arginine, Sigma), in 0.05 M Tris buffer, pH 7.5, containing 0.1 M NaCl (Folk et al., 1960). A 100- μ l aliquot of supernatant from the gut homogenate (diluted 1:3 with buffer) was added to 2.9 ml substrate-buffer mixture, and activity was monitored spectrophotometrically at 254 nm for 3 min. Carboxypeptidase B from porcine pancreas (Sigma) was used as a standard. The assay was replicated three times.

Endopeptidases. The determination of chymotrypsin activity required the use of 1 mM BTEE (benzoyl-L-tyrosine ethyl ester, Sigma; molar extinction coefficient of 964) (Hummel, 1959) in 50% MeOH mixed 1:1 with 0.04 M Tris buffer, pH 8.1, 0.01 M CaCl₂ (Walsh and Wilcox, 1970). A 25- μ l aliquot of supernatant from the gut homogenate (diluted 1:20 with buffer) was added to 2.9 ml of the substrate mixture, and enzyme activity was monitored spectrophotometrically at 256 nm for 3 min. Chymotrypsin from bovine pancreas (Sigma) was used as a standard. Each test solution was replicated at least three times.

For tryptic activity determination (Walsh, 1970), a 25- μ l aliquot of supernatant from the gut homogenate (diluted 1:20 with buffer) was added to 2.9 ml buffer (0.04 M Tris, pH 8.1, 0.01 M CaCl₂) containing 1.04 M TAME (*p*-

toluene-sulfonyl-L-arginine methyl ester, Sigma; molar extinction coefficient of 540) (Hummel, 1959). Tryptic activity was monitored spectrophotometrically at 247 nm for 3 min. Bovine trypsin (Sigma) was used as a standard. Each test solution was replicated at least three times.

Effect of Protease Inhibitors on Larval Proteolytic Activity. Inhibitors used for this study include: phenylmethylsulfonylfluoride (PMSF), which indicates the presence of serine at the active site (Gold, 1965); soybean trypsin inhibitor (STI), a naturally occurring trypsin inhibitor; *N*-tosyl-L-lysinechloromethyl ketone (TLCK), which indicates histidine at the active site of trypsin (Shaw, 1967); and L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK), which indicates histidine at the active site of chymotrypsin (Schoellman and Shaw, 1963). Solutions of individual inhibitors were prepared in 0.1 M phosphate buffer (for azocoll assays), or 1 mM HCl, and preincubated with an enzyme solution for 10 min at room temperature. Following this preincubation, the mixture was assayed for specific enzyme activity, using the appropriate substrate and buffer mixture (described above). Each assay was replicated at least three times.

Effect of Dietary Protein Concentration on Larval Growth

The effect of the concentration of dietary protein on the weight gain of larval *T. ni* and *P. rapae* was determined by allowing newly emerged neonate larvae to feed on control or test wheat germ-based diets until the majority of larvae feeding on the control diet developed to the second day of the fifth (ultimate) instar. Then all larvae, both controls and tests, were individually weighed. Each bioassay (20–40 larvae/cup; 3 cups/protein concentration; 4 protein concentrations/bioassay) for both species was replicated three times.

Statistics

All data were analyzed by the computer package SYSTAT (Systat, Inc., Evanston, Illinois) using the paired *t* test or ANOVA followed by orthogonal comparisons to determine the statistical significance at the 95% level.

RESULTS

General protease activity was readily detectable using a 1:3 dilution of the larval enzyme solution mixed with the substrate, azocoll. The rate of hydrolysis of azocoll by the diluted enzyme solution was significantly higher ($P < 0.001$, $r = 0.97$) for *P. rapae* than for *T. ni* (Table 1). [There was also a significant difference ($P < 0.001$, $r = 0.998$) between the rate of hydrolysis of azocoll by 80 $\mu\text{g/ml}$ of bovine trypsin and chymotrypsin (Table 1).]

There was no detectable exopeptidase (carboxypeptidase A or B) activity

TABLE 1. PROTEOLYTIC ACTIVITY IN MIDGUT LUMINAL FLUID FROM LARVAL *Pieris rapae* AND *Trichoplusia ni*

Enzyme	Substrate ^a	Activity
<i>P. rapae</i> (1:3 dilution)	Azocoll	104.4 ± 0.09 ^b
<i>T. ni</i> (1:3 dilution)	Azocoll	83.9 ± 0.04 ^b
Trypsin (80 µg/ml)	Azocoll	203.9 ± 0.2 ^b
Chymotrypsin (80 µg/ml)	Azocoll	165.1 ± 0.4 ^b
<i>P. rapae</i> (1:20 dilution)	TAME	13.5 ± 0.5 ^c
<i>P. rapae</i> (1:20 dilution)	BTEE	12.5 ± 2.8 ^c
<i>T. ni</i> (1:20 dilution)	TAME	26.3 ± 2.0 ^c
<i>T. ni</i> (1:20 dilution)	BETT	7.7 ± 0.7 ^c

^aSubstrates: azocoll = general proteolytic substrate; TAME = trypsin-specific substrate; BTEE = chymotrypsin-specific substrate.

^bActivity reported as mean change in OD/min ± SEM.

^cActivity reported as millimoles substrate hydrolyzed/min ± SEM.

in the guts of larval *T. ni* or *P. rapae*. However, the substrates for the endopeptidases, trypsin and chymotrypsin, were rapidly hydrolyzed by the midgut homogenates (diluted 1:20) prepared from these two species of insects (Table 1). Based on the rates of hydrolysis of the enzyme-specific substrates, the ratio of tryptic to chymotryptic activity for *T. ni* was 76:24, while the ratio for *P. rapae* was 52:48.

Enzyme activity relative to pH is shown in Figures 1 and 2. These data are reported as the rate of hydrolysis of substrate (millimoles per minute). For larval *T. ni* (Figure 1), optimal tryptic and chymotryptic activity occurs at pH 8.5 and 8.0, respectively. The optimal tryptic and chymotryptic activity from the luminal fluid of larval *P. rapae* (Figure 2) occurs at pH 8.0 and 9.0, respectively.

The ability to inhibit larval tryptic and chymotryptic activity was tested using specific inhibitors and was compared to the levels of inhibitor required to inhibit the activity of standard bovine trypsin and chymotrypsin. A 1 mg/ml solution of either STI or PMSF completely inhibited an 80 µg/ml solution of bovine trypsin (the uninhibited level of activity was 69 mmol TAME hydrolyzed/min), as measured by the TAME and azocoll assay (Table 2). However, the inhibition of tryptic activity from the guts of larval *T. ni* required 5 mg STI/ml to inhibit a 1:20 dilution of the enzyme solution by 92%, and 10 mg PMSF/ml inhibited the same enzyme solution by only 69% (Table 3), as measured by the TAME assay (uninhibited activity level was 33 mmol TAME hydrolyzed/min). When the 1:20 dilution of enzyme solution (tryptic activity producing

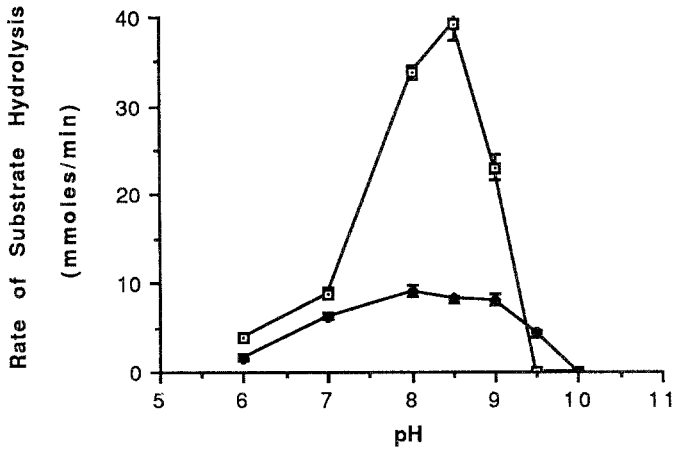


FIG. 1. The effect of pH on the proteolytic activity in the midgut luminal fluid from actively feeding fifth instar (ultimate) *Trichoplusia ni*. The open squares indicate tryptic activity; the solid diamonds indicate chymotryptic activity. The vertical lines indicate the 95% confidence interval. Each data point represents at least three replicates.

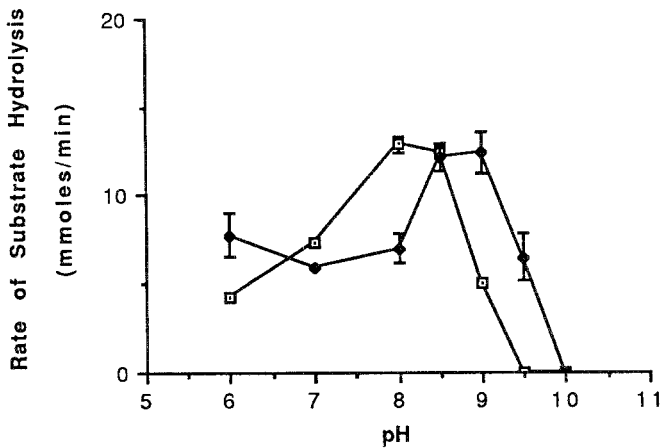


FIG. 2. The effect of pH on the proteolytic activity in the midgut luminal fluid from actively feeding fifth instar (ultimate) *Pieris rapae*. The open squares indicate tryptic activity; the solid diamonds indicate chymotryptic activity. The vertical lines denote the 95% confidence interval. Each data point represents at least three replicates.

TABLE 2. EFFECT OF PROTEASE INHIBITORS ON BOVINE TRYPSIN AND CHYMOTRYPSIN

Enzyme and substrate	Inhibitor ^a	Ratio, enzyme to inhibitor	Mean % inhibition
Trypsin (80 µg/ml) and TAME	20 µg STI/ml	1:1	0.45
Trypsin (80 µg/ml) and TAME	40 µg STI/ml	1:1	7.2
Trypsin (80 µg/ml) and TAME	80 µg STI/ml	1:1	57.2
Trypsin (80 µg/ml) and TAME	1 mg STI/ml	1:1	100
Trypsin (80 µg/ml) and TAME	1 mg PMSF/ml	1:1	100
Trypsin (80 µg/ml) and Azocoll	1 mg STI/ml	1:1	99.9
Chymotrypsin (80 µg/ml) and Azocoll	1 mg TPCK/ml	1:1	95.9

^aProtease inhibitors: STI, TLCK, and PMSF are trypsin inhibitors; TPCK and PMSF are chymotrypsin inhibitors.

13.5 mmol TAME hydrolyzed/min) from larval *P. rapae* was mixed 1:1 with 5 mg STI/ml, tryptic activity was only reduced by 35% (Table 4). A similar mixture of enzyme and inhibitor, replacing the STI with 10 mg PMSF/ml, resulted in a 53% reduction of tryptic activity. Thus, when comparing the abil-

TABLE 3. EFFECT OF PROTEASE INHIBITORS ON ENZYME ACTIVITY FROM FIFTH INSTAR GUT LUMEN OF *Trichoplusia ni*

Enzyme solution ^a	Substrate	Inhibitor ^b	Ratio, enzyme to inhibitor	Mean % inhibition
1:3 w/P-8	Azocoll	1 mg PMSF/ml	1:2	69.2
1:3 w/P-8	Azocoll	1 mg TPCK/ml	1:2	72.0
1:3 w/P-8	Azocoll	1 mg TLCK/ml	1:2	86.4
1:20 w/P-8.5	TAME	1 mg PMSF/ml	1:1	30.1
1:20 w/P-8.5	TAME	5 mg PMSF/ml	1:1	62.5
1:20 w/P-8.5	TAME	10 mg PMSF/ml	1:1	68.6
1:20 w/P-8	BTEE	10 mg PMSF/ml	1:1	100
1:15 w/P-8	TAME	0.06 mg STI/ml	1:1	64.7
1:15 w/P-8	TAME	0.5 mg STI/ml	1:1	69.9
1:15 w/P-8	TAME	1 mg STI/ml	1:1	68.6
1:15 w/P-8	TAME	1 mg STI/ml	1:2	80.8
1:15 w/P-8	TAME	1 mg STI/ml	1:3	83.1
1:15 w/P-8	TAME	1 mg STI/ml	1:5	81.9
1:15 w/P-8	TAME	5 mg STI/ml	1:1	87.2
1:20 w/P-8	TAME	5 mg STI/ml	1:1	92.0

^aDilution factors of enzyme solutions are presented as a ratio of enzyme to buffer. Buffer solutions: P-8 = 0.1 M phosphate buffer, pH 8.0; P-8.5 = 0.1 M phosphate buffer, pH 8.5.

^bProtease inhibitors: PMSF, TLCK, and STI are trypsin inhibitors; TPCK and PMSF are chymotrypsin inhibitors.

TABLE 4. EFFECT OF PROTEASE INHIBITORS ON TRYPTIC AND CHYMOTRYPTIC ACTIVITY IN LUMINAL FLUID FROM ACTIVELY FEEDING FIFTH INSTAR *Pieris rapae*

Enzyme solution ^a	Substrate	Inhibitor ^b	Ratio, enzyme to inhibitor	Mean % inhibition
1:3 w/P-8	Azocoll	1 mg PMSF/ml	1:2	77.3
1:3 w/P-8	Azocoll	1 mg TPCK/ml	1:2	77.7
1:3 w/P-8	Azocoll	1 mg TLCK/ml	1:2	88.9
1:20 w/P-8	TAME	5 mg STI/ml	1:1	34.8
1:20 w/P-8	TAME	10 mg PMSF/ml	1:1	52.8
1:20 w/P-8	BTEE	10 mg PMSF/ml	1:1	100
1:20 w/P-8.5	BTEE	10 mg PMSF/ml	1:1	100

^aDilution factor of enzyme solutions are presented as a ratio of enzyme:buffer. Buffer solutions: P-8 = 0.1 M phosphate buffer, pH 8.0; p-8.5 = 0.1 M phosphate buffer, pH 8.5.

^bProtease inhibitors: PMSF, TLCK, and STI are trypsin inhibitors; TPCK and PMSF are chymotrypsin inhibitors.

ity of the selected inhibitors to inhibit larval tryptic activity with that of the inhibition of bovine trypsin, even 5–10 times the amount of inhibitor that would result in 100% inhibition of bovine tryptic activity does not completely eliminate larval tryptic activity.

Bovine chymotryptic activity (80 μ g/ml resulted in hydrolysis of 30 mmol BTEE/min) was inhibited by 95.9% by the addition (1:1 mixture) of 1 mg TPCK/ml (azocoll assay) (Table 2). The mixture of *T. ni* (uninhibited activity was 54.5 mmol BTEE hydrolyzed/min) or *P. rapae* (uninhibited activity was 145 mmol BTEE hydrolyzed/min) enzyme solution (diluted 1:3) with a similar concentration of TPCK resulted in a 72% or 77% reduction in activity, respectively (Tables 3 and 4). A 10 mg/ml preparation of PMSF completely inhibited the chymotryptic activity for the two test species of insect (Tables 3 and 4).

For both species of Lepidoptera, there is a significant positive linear relationship between the concentration of dietary protein and larval weight (*P. rapae* slope = 27.2, $r = 0.93$, $P < 0.001$; *T. ni* slope = 30.4, $r = 0.70$, $P < 0.001$) (Figure 3). In addition, larval *P. rapae* shows a significantly greater reduction in weight gain with the reduction of dietary protein concentration than *T. ni* ($P < 0.001$).

DISCUSSION

Larval lepidopteran digestive physiology is generally characterized by an alkaline midgut and the secretion of endopeptidases for luminal midgut proteolytic degradation (Broadway and Duffey, 1986a; Sasaki and Suzuki, 1982; Law

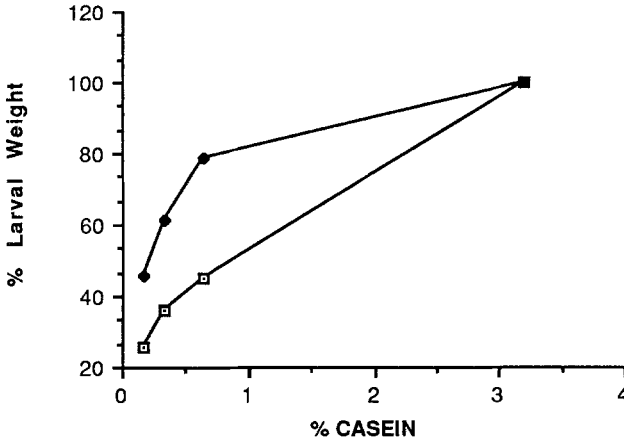


FIG. 3. The effect of dietary protein concentration on the weight gain of larval *Pieris rapae* and *Trichoplusia ni*. The solid diamonds indicate growth of larval *T. ni*; the open squares indicate growth of larval *P. rapae*. The vertical lines indicate the standard error of the means. Each data point represents at least three replicates with a total of 170–350 larvae.

et al., 1977; Ward, 1975; Miller et al., 1974; Ishaaya et al., 1971). Larval *Trichoplusia ni* and *Pieris rapae* follow this general trend, exhibiting alkaline midguts (Broadway, unpublished data; Berenbaum, 1980), and proteolytic activity dominated by serine endopeptidases (trypsin- and chymotrypsin-like activity) (Table 1), with optimal enzyme activity at pH 8–9 (Figures 1 and 2). There was no detectable exopeptidase activity in the larval midguts of either species.

The results of the general in vitro protease assay (Tables 3 and 4) demonstrated that 86–89% of the larval enzyme activity could be eliminated by the addition of tryptic or chymotryptic inhibitors, indicating that trypsin and chymotrypsin are the major, if not sole, enzymes responsible for protein digestion. The data from enzyme-specific assays indicated that the chymotryptic activity in the midgut fluid from both species of Lepidoptera could be completely inhibited with selected inhibitors, but the insect tryptic activity could not be completely eliminated, even at extremely high concentrations of appropriate inhibitors (Tables 3 and 4). This inability to completely eliminate larval tryptic activity suggests that (1) the inhibitors form a weak bond with the insect trypticlike enzyme and/or (2) the insect trypticlike activity originates from more than one protein, resulting in complete inhibition of the enzyme(s) susceptible to the inhibitor and reduced or no inhibition of the less sensitive enzyme(s).

Although the digestive physiology of larval *P. rapae* and *T. ni* are similar

in many respects, there are striking differences when comparing the results from the in vitro digestion assays. One difference is the relative proportion of tryptic to chymotryptic activity in the midgut fluid (Table 1). The midguts of larval *P. rapae* contain a 1 : 1 mixture of tryptic and chymotryptic activity, whereas larval *T. ni* have more than three times as much tryptic activity compared to chymotryptic activity. This information suggests that these two species of Lepidoptera have different strategies of digestion of dietary protein. Trypsins are serine proteinases that cleave intact protein chains on the carboxyl side of basic L-amino acids such as arginine or lysine, whereas chymotrypsins cleave intact protein chains on the carboxyl side of aromatic amino acids (Walsh and Wilcox, 1970). Broadway and Duffey (1988) have shown that, for lepidopterous larvae in which trypsin is the primary protease, there is a significant positive correlation between larval growth and the relative amount of arginine + lysine residues in the dietary protein. Conversely, one may surmise that a mixture of different proteases (each acting at different sites on a protein) will digest dietary protein more efficiently and/or rapidly than a single protease. Following this line of reasoning, the relative proportions of different proteases in the gut will determine the relative number of amino acid sites at which proteins can be cleaved (or the extent of digestion) and, ultimately, the availability of proteinaceous nutrients. This line of reasoning is supported by the data demonstrating that the level of general protease activity in the midgut fluid (as measured in vitro by the rate of hydrolysis of azocoll) is significantly higher in larval *P. rapae* than *T. ni* (Table 1). This ability of larval *P. rapae* to digest protein more efficiently than *T. ni* may be related to the higher level of dietary protein required by *P. rapae*, as demonstrated by the protein bioassays (Figure 3).

Thus, for the two species of Lepidoptera examined in the current study, the protease activity profile (the total tryptic + chymotryptic activity, and the relative proportion of each enzyme), and the rate of in vitro digestion of azocoll provide an insight into the strategy and efficiency of proteolysis during the initial (luminal) phase of digestion. *P. rapae* and *T. ni* have equivalent total tryptic + chymotryptic activity; however, the proportions of enzymes are different. Using this equivalent amount of total enzyme activity, *P. rapae* digests azocoll at a significantly faster rate than *T. ni*, suggesting that *P. rapae* digests protein more efficiently than *T. ni*. In addition, the tryptic and chymotryptic activity from the midgut fluid of *P. rapae* is less sensitive to specific enzyme inhibitors than is *T. ni*. This suggests that *P. rapae* would digest a natural protein-proteinase inhibitor mixture more effectively than *T. ni*. This is physiologically adaptive, since the oligophagous larval *P. rapae* has a significantly higher dietary protein requirement than polyphagous larval *T. ni*.

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TEMPORAL CHANGES IN COLONY CUTICULAR
HYDROCARBON PATTERNS OF *Solenopsis invicta*
Implications for Nestmate Recognition

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Abstract—Heritable cuticular hydrocarbon patterns of *Solenopsis invicta* workers are consistent within colonies for a given sampling time but vary sufficiently from colony to colony to distinguish the colonies from each other. In addition, cuticular hydrocarbon patterns change within colonies over time. Nestmate recognition cues found on the individual's cuticle, can be from heritable or environmental sources, and are a subset of colony odor. The cuticular hydrocarbons can be used as a model for heritable nestmate recognition cues. We propose that because potential nestmate recognition cues, both environmental and genetic, are dynamic in nature rather than static, during its lifetime a worker must continually update its perception (template) of colony odor and nestmate recognition cues.

Key Words—*Solenopsis invicta*, Hymenoptera, Formicidae, cuticular hydrocarbons, nestmate recognition, colony odor, temporal changes.

INTRODUCTION

Cuticular hydrocarbons serve several purposes in insects, such as prevention of desiccation and regulation of cuticular permeability (Hadley, 1980). In addition, this class of chemically inert compounds can have many semiochemical

functions, such as alarm, recruitment, defense, sex attractants, and host attractants (see Howard and Blomquist, 1982, for review).

Solenopsis invicta Buren cuticular hydrocarbons constitute the largest lipid class (over 70%) on the cuticle (Lok et al., 1975) and are species specific (Nelson et al., 1980; Vander Meer, unpublished). *S. invicta* and *S. richteri* have the same cuticular hydrocarbons, identified as normal, monomethyl, and dimethyl branched alkanes (Lok et al., 1975; Nelson et al., 1980). However, quantitative patterns for the two species are distinctly different (Nelson et al., 1980; Vander Meer et al., 1985). Their species specificity has been used as a reliable chemotaxonomic tool to attack several problems (Vander Meer, 1986). The heritable nature of these compounds has been clearly demonstrated in studies of hybridization between *S. invicta* and *S. richteri* (Vander Meer et al., 1985; Ross et al., 1987).

It is generally accepted that nestmate recognition in social insects involves the detection of specific odiferous cues on the cuticle (Wilson, 1971). Heritable (Mintzer and Vinson, 1985) and/or environmental (Jutsum et al., 1979) odors can be operational in ant nestmate recognition. Odors under genetic control or resulting from genetic-environment interactions are termed heritable odors. It has been demonstrated that *S. invicta* uses both heritable and environmental nestmate recognition cues (Obin, 1986; Obin and Vander Meer, 1989). Cuticular hydrocarbons have been implicated in species and caste recognition in termites (Howard et al., 1982a), and several species of termitophiles have been shown to have cuticular hydrocarbons identical to their host, which was associated with their integration into the host colony (Howard et al., 1982b). Hydrocarbons have also been implicated in nestmate recognition of the ant *Camponotus vagus* (Bonavita-Cougourdan et al., 1987).

Although there is no direct evidence for the use of cuticular hydrocarbons in nestmate recognition in *S. invicta*, we have used the cuticular hydrocarbons as a model to study the quantitative variation of heritable components of colony odor. We report here that individual colonies have distinguishable cuticular hydrocarbon patterns; however, these patterns change with time. The implications of the dynamic nature of these heritable characters in relationship to nestmate recognition mechanisms are discussed.

METHODS AND MATERIALS

Source of Colonies. Laboratory colonies of *S. invicta* were reared from newly mated queens collected near Gainesville, Florida, or Gulfport, Mississippi, and reared in the Gainesville laboratory by the procedures described by Banks et al. (1981). Workers from monogynous field colonies were sampled

from the Gainesville, Florida, area. *S. invicta* workers undergo age-related polyethism and have been categorized as nurses, reserves, and foragers (Miranda and Vinson, 1981). Only reserve workers were used in this study. Reserve workers were defined in laboratory colonies as those workers that were inactive and clustered together at the outside of the colony cell (Sorensen et al., 1981). For field colonies, reserve workers were obtained by placing a beaker (50 ml), coated on the inside lip with Fluon, partly into the surface of a mound. If any workers were observed carrying brood, the sample was discarded. In the laboratory, the collected ants were anesthetized with CO₂, and three replicate samples of 50 or 100 reserve workers were placed in a vial and weighed on a Mettler H51Ar analytical balance. Worker ants within a range of 1.5–4.0 mm in length, readily determined by visual inspection, were used for the analysis of cuticular hydrocarbons.

Analysis of Cuticular Hydrocarbons. Cuticular hydrocarbons were obtained by soaking the ant samples for 7 min in hexane (ca. 2 ml HPLC grade, Baker Chemical Co., Phillipsburg, New Jersey). The samples were gently shaken for the first and last 15 sec of the soak period. Immediately after the rinses were complete, 9 μ l of a 0.1% hexane solution of *n*-pentacosane was added for quantitation as an internal standard. Samples were evaporated to a small volume under a stream of nitrogen and the residue applied to a Pasteur pipet silicic acid HA column (325 mesh particle size, Bio Rad, Richmond, California). Hydrocarbons were isolated by eluting the column with hexane (Christie, 1973). The hexane eluate was evaporated to ca. 50 μ l and an aliquot was analyzed on a Varian 3700 gas chromatograph (Varian Associates, Sunnyvale, California) equipped with a flame-ionization detector. A 1.8-m \times 2-mm-ID glass column packed with OV-17 on 120–140 mesh Gas Chrom Q (Applied Science Laboratories, Deerfield, Illinois) was used to separate the hydrocarbons. The oven was programmed from 175°C to 235°C at 3°C/min. Qualitative and quantitative data were obtained for the five major hydrocarbon peaks (Figure 1) using a Varian Vista 401 data processor (Varian Associates).

Quantitative Influence of Soak Time. Three replicates of 100 workers each from the same colony were weighed and allowed to soak in hexane, containing *n*-pentacosane as an internal standard, for the following time periods: 3, 5, 7, 10, 15 min, and overnight. Aliquots of the hexane were removed at the indicated time periods, and hydrocarbons were isolated and analyzed as described above.

Analysis of Data. The individual peak areas were quantitated using the internal standard and digitalized as the number of nanograms per sample. The data were normalized using the weight of the corresponding ant sample. We used principal component analysis, a pattern recognition technique (Wold, 1976), to establish relationships between the GC profiles of the ants and several

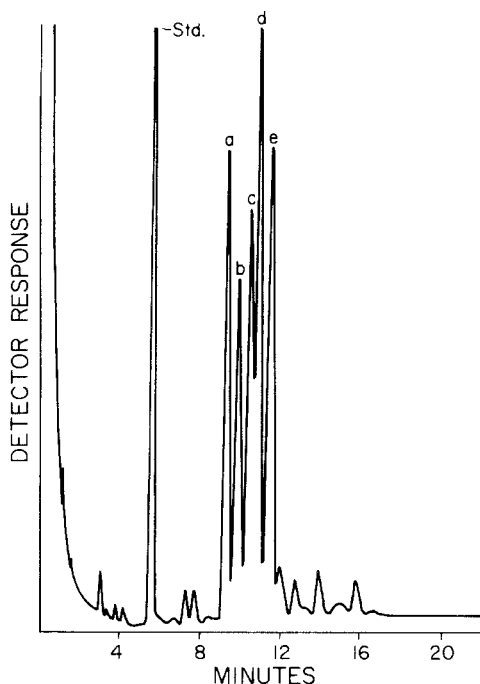


FIG. 1. Gas chromatogram of cuticular hydrocarbons from *Solenopsis invicta* workers. Peak a: n-heptacosane, b: 13-methylheptacosane, c: 13,15-dimethylheptacosane, d: 3-methylheptacosane, and e: 3,9-dimethylheptacosane.

variables, such as colony source and time. The principal component projections were made without the use of information about the class assignment of the samples.

Hydrocarbon Time Study. Four laboratory colonies were selected for a study to determine whether hydrocarbon patterns varied with time. These colonies were sampled, as described above, three to six times during a four- to eight-month period. The hydrocarbons were isolated, separated by gas chromatography, and the data analyzed as described in the previous sections.

RESULTS

The quantitative results for the removal of cuticular hydrocarbons over several soak times is shown in Table 1. The percent composition of the five hydrocarbon peaks under consideration varied most for time periods of 3 min and overnight. Consistent results were obtained for time periods 5, 7, 10, and

TABLE 1. EFFECT OF SOAK TIME ON EXTRACTIONS OF HYDROCARBONS FROM *S. invicta* WORKER ANTS

Soak time	Percent hydrocarbon peak ^a					Hydrocarbon (μ g)	
	A	B	C	D	E	Per mg ant	Per 100 ants
3 min	12.4 \pm 0 ^b	27.6 \pm 0.2	18.6 \pm 0.0	15.4 \pm 0.0	25.9 \pm 0.2	0.62 \pm 0.02	31.8 \pm 1.0
5 min	11.6 \pm 0.6	27.8 \pm 0.2	19.2 \pm 0.1	15.2 \pm 0.0	26.1 \pm 0.3	0.76 \pm 0.11	38.7 \pm 6.7
7 min	10.4 \pm 0.2	27.3 \pm 0.2	19.1 \pm 0.3	15.6 \pm 0.1	27.7 \pm 0.6	0.76 \pm 0.04	37.3 \pm 2.5
10 min	10.3 \pm 0.3	27.8 \pm 0.2	19.8 \pm 0.2	15.2 \pm 0.1	27.0 \pm 0.2	0.80 \pm 0.01	39.8 \pm 2.5
15 min	10.0 \pm 0.0	27.1 \pm 0.3	19.8 \pm 0.0	15.4 \pm 0.2	27.8 \pm 0.0	0.88 \pm 0.04	46.4 \pm 4.2
Overnight	8.7 \pm 0.4	26.0 \pm 1.0	22.4 \pm 2.4	15.6 \pm 0.4	27.2 \pm 0.6	2.91 \pm 0.27	138.8 \pm 14.2

^aSee Figure 1.

^bMean \pm standard deviation (N = 3).

15 min. However, the quantity extracted, whether expressed as micrograms per milligram of ant or micrograms per 100 ants, was most consistent for the 5-, 7-, and 10-min soak periods. Consequently, all subsequent samples were soaked for 7 min.

The inter- and intracolony variations in cuticular hydrocarbon patterns were monitored from samples collected within two weeks of each other. A principal component analysis of a set of 27 ant samples from nine different laboratory and field colonies (three replicates each taken at the same time) was performed using the five major hydrocarbon GC peaks as descriptors (Figure 1). A plot of the first two principal components is shown in Figure 2. The first two eigenvectors account for 85.9% of the total cumulative variance.

Pattern groupings according to each individual colony are evident. As determined by inspection (Figure 2), within-colony variation is less than the between-colony variation. There were no obvious principal component plot separations based on a colony's geographic origin (Mississippi and Florida), nor was there a separation of field versus laboratory colonies.

Cuticular hydrocarbon patterns of laboratory colonies sampled at several

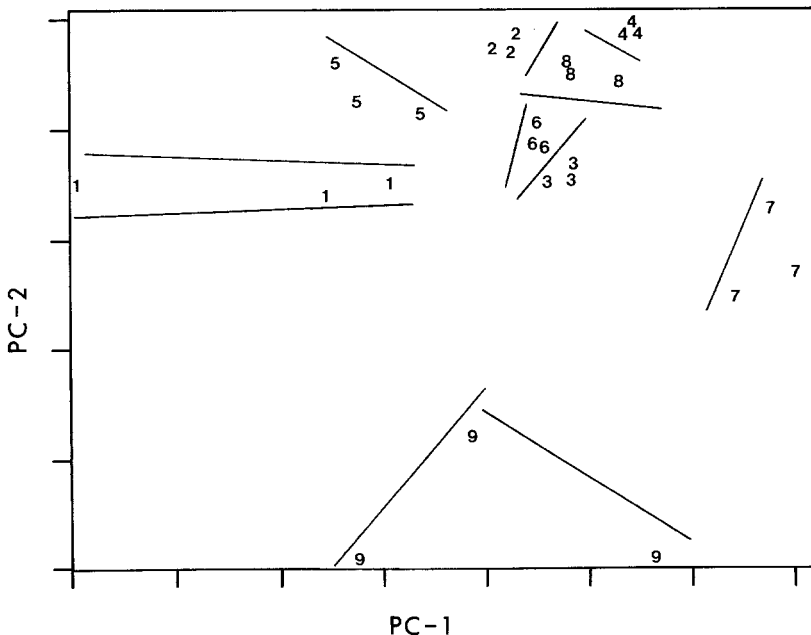


FIG. 2. A plot of the first two principle components of the five major hydrocarbons for nine *S. invicta* colonies. Colonies 1-4 are field collected from Florida; colonies 5-7 are lab-reared in Gainesville, Florida, but originated from newly mated queens from Gulfport, Mississippi; colonies 8 and 9 are lab-reared in Gainesville, Florida, collected in Florida from newly mated queens.

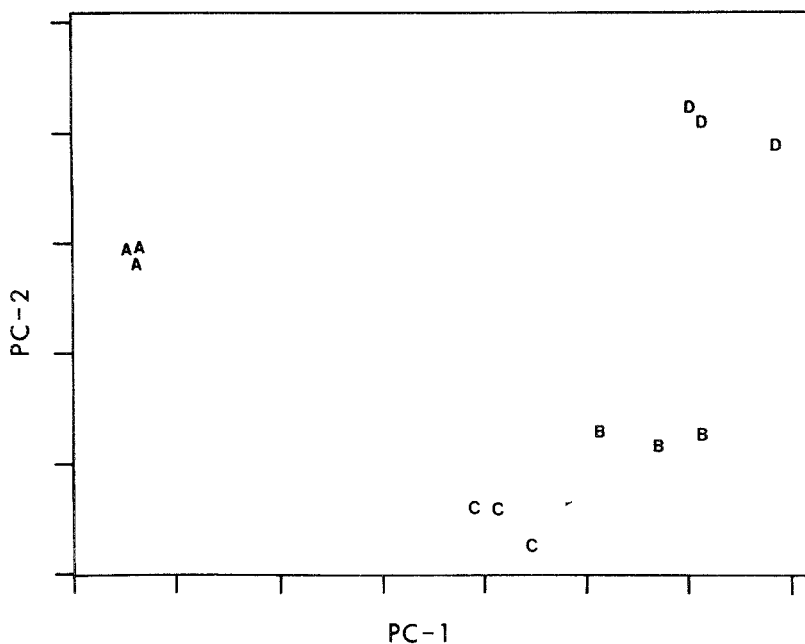


FIG. 3. A plot of the first two principle components of the five major cuticular hydrocarbons collected from field colony 3 (Figure 2) over four time periods. The letters A, B, C, and D represent collections in November 1982, January 1983, February 1983, and April 1983, respectively.

time periods were not static. Principal component maps of the first two principal components developed from the five GC peaks showed pattern groupings according to the date of collection in all four cases. The variation in replicates within a time period was less than that between time periods. An example of one colony sampled at four different times is shown in Figure 3. The first two eigenvectors account for 90% of the total cumulative variance. Use of an internal standard and careful attention to the proper peak integration baselines minimized instrument variation. When a single principal component plot was made of several colonies at several times, the pattern fluctuation of a colony with time caused overlap with other colony patterns.

DISCUSSION

Hydrocarbon Variability. It is not possible to obtain a rinse or soak of whole ants that does not contain compounds from a variety of sources other than the cuticle (Vander Meer, 1989). For example, species-specific fire ant venom alkaloids and hydrocarbons are routinely obtained by simply soaking

worker ants in hexane (Vander Meer et al., 1985; Ross et al., 1987). In this study, hydrocarbons were isolated by column chromatography to avoid contamination from other classes of chemicals. Quantitative analysis of the hydrocarbons from worker ants soaked for various times showed a dramatic increase in material when samples were soaked overnight. This probably reflects leaching of the large amount of hydrocarbon found in the postpharyngeal gland (Vander Meer et al., 1982). This material can exit the gland via the pharynx and mouth parts. A 7-min soak time was used for all subsequent analyses because of the consistent results from 5–10 min and to provide the best approximation of a cuticular hydrocarbon profile.

The separation of nine *S. invicta* colonies from diverse locations at a single collection time (Figure 2) is indicative of the quantitative intercolony variability possible in the cuticular hydrocarbons. These data also suggest that cuticular hydrocarbon variation is random throughout the distribution of *S. invicta* in the United States, since there was no obvious separation of colonies based on whether they were derived from Florida or Mississippi. Nestmate recognition studies (Obin, 1986) support this data.

The lack of separation of field-collected colonies from laboratory-maintained colonies in the principle component plots suggests that diet and other environmental factors do not have a noticeable influence on the phenotypic expression of cuticular hydrocarbon patterns (no gene–environment interaction). Similar results for hydrocarbon analysis of laboratory and field colonies were obtained by Obin (1986). The genetic origin of these compounds has been confirmed by the concordance of hydrocarbon profile data and genetic data (isozymes) in differentiating hybrid fire ants from their *S. invicta*–*S. richteri* parents (Ross et al., 1987).

It should be noted that the principal component projections (Figures 2 and 3) were made without the use of information about the class assignment of the samples. Therefore, distinct separations are a strong indication of real differences in the GC profiles of the ants.

The analysis of colony hydrocarbons over time demonstrated that individual colony profiles (reserve worker temporal subcaste) are dynamically changing, although for any given time period the profiles for the three replicates of a given colony are consistent (Figure 3). This is in part because the social interactions of the colony workers ensure the efficient transfer of cuticular chemicals (environmental and genetic) throughout the worker force of a colony. The transfer of compounds on the cuticle of several ant species has been clearly demonstrated by: (1) the mixing of species-specific cuticular chemical components on individuals from laboratory colonies composed of two different species (Errard and Jallon, 1987; Vander Meer, unpublished), (2) the rapid distribution of radiolabeled markers in fire ant colonies (Sorensen et al., 1985), and (3) the transfer of species-specific fire ant hydrocarbons to a myrmecophilous beetle

(Vander Meer and Wojcik, 1982). These interactions decrease the variability of all chemicals found on the worker surface and confound studies aimed at the investigation of individual variation. Although in this study we cannot address the mechanism of profile change, we can definitively state that heritable cuticular hydrocarbon profiles undergo continuous quantitative changes during the life of a colony. The magnitude of the temporal changes creates overlap with the clusters of other colony patterns.

Nestmate Recognition. Nestmate recognition occurs in ants when a worker distinguishes between workers of another colony and its own colony following a pause and sweep of the antennae over the others' body (see Breed and Bennett, 1987, for a review). The odor on the surface of a worker is a composite of environmentally and genetically derived components. In general, the odors used by a particular species or colony for nestmate recognition are a subset of all possible odors and may be composed of any combination of components (Vander Meer, 1988). *S. invicta* uses both environmental and heritable nestmate recognition cues (Obin, 1986; Obin and Vander Meer, 1988). Studies with *S. invicta* have investigated the effects of environmental components on nestmate recognition (Obin and Vander Meer 1988, 1989). However, although cuticular hydrocarbons are strongly correlated with nestmate recognition in *Camponotus vagus* (Bonavita-Cougourdan et al., 1987) and associated with the integration and survival of a myrmecophilous beetle in fire ant nests (Vander Meer and Wojcik, 1982), the composition of the heritable part of the cues has not been determined. In any event, cuticular hydrocarbons are a part of the heritable component of colony odor and can be used as a general model for heritable nestmate recognition cues. This paper establishes the dynamic nature of these compounds.

Nestmate recognition cues are only half of the nestmate recognition process. It is generally considered that colony-specific chemical patterns or templates (Breed and Bennet, 1987) are learned by workers soon after eclosion (Morel, 1983; Errard, 1984; Morel et al., 1988). Although newly eclosed workers may not be completely olfactorally naive in terms of nestmate recognition (Isingrini et al., 1985), they learn to recognize the first odors they experience, whether from their own colony or, in the case of laboratory adoption experiments, from another colony or species (Carlin and Hölldobler, 1983; Errard, 1986; Morel and Blum, 1988).

Our results indicate that early template formation must be modified by continuous updating. The dynamic nature of the environmental part of colony odor is obvious. The variation in cuticular hydrocarbons demonstrates that the heritable component of colony odor is not fixed either. Because of the dynamic nature of colony odor and, by implication, nestmate recognition cues, we propose that workers are not hard-wired to a specific chemical or pattern of chemicals but have a great deal of flexibility through a process of continuous, iterative

learning that provides a mechanism for colony members to cope with a constantly changing chemical environment, as well as within-colony individual genetic variations (hydrocarbon patterns vary temporally as widely as between colony patterns). This concept was alluded to by Wallis (1963), who likened the recognition process to odor habituation and suggested that an ant "is continuously habituating to the slight variations in the odour of its nest-mates." Our work supports the necessity for such a process in nestmate recognition.

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EFFECT OF JUGLONE ON FRESHWATER ALGAL GROWTH

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Abstract—Allelopathic growth inhibition of various freshwater algal species was observed upon the addition of juglone to culture medium under laboratory conditions. Juglone is a phenolic chemical compound common to most parts of the black walnut (*Juglans nigra* L.). Growth inhibition of all species occurred at a juglone concentration of 10^{-3} M. Three of the five species were inhibited by a concentration of 10^{-4} M juglone, while no inhibition occurred at 10^{-5} M juglone. The results of this study indicate that juglone is less toxic to algae than fish. Therefore, the potential of juglone for aquatic management purposes is limited. Algal biomass will increase at natural juglone concentrations from 10^{-8} to 10^{-4} M due to the elimination of secondary producers. Algal biomass will decrease at natural juglone concentrations between 10^{-4} and 10^{-3} M. Species richness will decrease at juglone concentrations of 10^{-5} and higher.

Key Words—Juglone, allelopathy, black walnut, *Juglans nigra* L., freshwater algae, algal biomass.

INTRODUCTION

Allelopathy is any direct or indirect harmful effect by one plant on another through the production of chemical compounds that escape into the environment (Rice, 1974). Allelochemicals belong to a few major groups of compounds among secondary plant substances, including terpenoid substances, steroids,

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alkaloids, organic cyanides, flavonoids, phenolic acids, and other aromatic compounds (Whittaker and Feeny, 1971). Many instances of allelopathy have been reported for a variety of plants (reviewed in Rice, 1974; Waller, 1987). Both Putnam and Duke (1978) and Rice (1979) suggested that allelopathic compounds could be utilized for biological weed control. Many studies have since examined the potential for agronomic weed control by allelopathic crops (Alsaadawi et al., 1986; Lodhi et al., 1987; Leather, 1987).

Robson (1977) suggested that allelopathy be used for the control of aquatic weeds. Many studies have examined the effects of allelochemicals from aquatic plants upon other aquatic plants. Eelgrass, *Zostera marina* L., was found to inhibit epiphytic and nearby algae and bacteria (Harrison and Chan, 1980). Similarly, marsh grass, *Spartina alterniflora*, and water shield, *Brasenia schreberi* Gmelin, were each shown to possess some allelopathic agent against various species of algae (Valiela et al., 1979; Elakovich and Wooten, 1987). Elakovich and Wooten (1987) suggested that water shield may, therefore, be useful for aquatic plant management.

Allelopathic interactions existing between diatoms and cyanobacteria were found to be one of the most significant controlling factors in the determination of their bloom sequence (Keating, 1977). Based upon these interactions, Keating (1977, 1978) discussed the potential of allelopathy for lake management. She described how the use of algicidal allelopathic species would be extremely advantageous in comparison with the undesirable application of copper sulfate to the lake environment.

However, Szczepanski (1977) suggested that the simple replacement of aquatic weeds with other strongly allelopathic plants would simply replace one problematic species with another. Therefore, allelopaths must be used that will not become weedy themselves. Plants that are not located within the immediate aquatic environment, yet would release allelochemicals into the system, would be ideal for this purpose.

Armstrong and Boalch (1961) and McLachlan and Cragie (1964) showed that phenolic compounds accumulate in marine waters. Three phenolic compounds released from three species of marine algae (*Polysiphonia morrowii*, *P. lanosa*, and *Monostroma fuscum*) were subsequently found to be highly toxic to several species of unicellular marine algae (McLachlan and Cragie, 1966). This suggests that phenolic compounds possess qualities that may be useful for the control of eutrophyng algae.

Black walnut (*Juglans nigra* L.) is known to cause allelopathic effects in a wide variety of organisms (Rice, 1974). Stickney and Hoy (1881) were the first to report that vegetation under black walnut is very sparse compared with that under most shade trees. The phenolic compound, juglone (5-hydroxy-1,4-naphthoquinone), was suggested by Massey (1925) to be the toxic constituent of black walnut. This was later confirmed by Davis (1928). Many studies have

since described the allelopathic effects of this chemical upon a variety of both plant and animal species (Brooks, 1951; Westfall et al., 1961; MacDaniels and Pinnow, 1976).

Juglone is present in the greatest quantities very near the tree (Ponder and Tadros, 1985). The concentration decreases with both soil depth and distance from the tree. Thus, the formation of a range of the presence of juglone must result. Should either a lake or stream be located within this range, the dispersal of juglone into the aquatic system will occur.

The majority of studies of the inhibitory effects of juglone have involved examinations upon terrestrial organisms. Furthermore, of the studies that have been performed upon aquatic organisms, only the effects upon animal species have been examined (Westfall et al., 1961; Marking, 1970). There have been no studies examining the effects of juglone upon aquatic plant species.

The purpose of this study was to determine the effects of juglone concentration upon the growth of freshwater algae.

METHODS AND MATERIALS

The effect of varying concentrations of juglone upon the growth of five species of freshwater algae was examined. Chlorophytes were chosen due to their large distribution and tremendous production capabilities in the natural environment. The species chosen were *Closterium acerosum* (Schrank), *Pandorina morum* Bory, *Micrasterias thomasiana* Arch., *Spirogyra grevilleana* (Hass.), and *Eudorina californica* (Shaw). All were easily obtainable (Carolina Biological Supply, Burlington, North Carolina) and common to the lakes and streams of North America.

The algae were cultured in Alga-Gro Freshwater Medium (Carolina Biological Supply) containing juglone (Aldrich Chemical Co., Milwaukee, Wisconsin) in concentrations of 0, 10^{-5} , 10^{-4} , and 10^{-3} M. The cultures were maintained in 500-ml Erlenmeyer flasks at $25 \pm 1^\circ\text{C}$ with a 16-hr light to 8-hr dark cycle under a lighting bank of Sylvania 40-W cool white fluorescent lights providing an intensity of ca. 300 foot-candles.

To ensure an even distribution of cell concentration before sampling, each culture was mechanically shaken at 350 rpm for 10 min. Two replicate samples of 5 ml each were taken from each culture daily for two weeks. These were pipetted into centrifuge tubes and centrifuged at 3000 rpm for 10 min. The pellets were placed into preweighed crucibles, dried overnight at 100°C , and weighed to 0.001 g. Dry weight (biomass) per volume was determined for each sample. Statistical analysis of treatment biomasses from control biomasses at each juglone concentration was performed using ANOVA.

RESULTS

Biomasses of all algal species tested were significantly reduced by a juglone concentration of 10^{-3} M (Table 1). In contrast, a concentration of 10^{-5} M juglone was not inhibitory to any species tested. A juglone concentration of 10^{-4} M was inhibitory to three of the five species tested. This indicates that the concentration of juglone within a natural aquatic system must reach approximately 10^{-4} M for algal inhibition to occur. No correlation existed between the taxonomic position of the algal species within Chlorophyta and the degree of inhibition observed. In addition, no correlation existed between the condition of the aquatic system to which the alga is most common (i.e., oligotrophic, mesotrophic, or eutrophic) and the degree of inhibition.

A juglone concentration of 10^{-3} M caused growth reductions of 83–89% (Table 2). Table 2 also illustrates that the reduction in growth caused by a 10^{-4}

TABLE 1. EFFECT OF JUGLONE CONCENTRATION ON ALGAL GROWTH

Species	Mean biomass/volume (g/liter) ^a			
	0 M	10^{-5} M	10^{-4} M	10^{-3} M
<i>C. acerosum</i>	0.859	0.954	0.646*	0.139**
<i>E. californica</i>	0.928	0.945	0.797	0.130**
<i>M. thomasiana</i>	1.02	1.14	0.890	0.115**
<i>P. morum</i>	1.04	0.972	0.732*	0.180**
<i>S. grevilleana</i>	0.921	0.948	0.729*	0.112**

^a Means of experimental cultures significantly different by ANOVA from control culture are indicated by * for $P \leq 0.05$ and by ** for $P \leq 0.01$.

TABLE 2. EFFECT OF JUGLONE CONCENTRATION OF MEAN TREATMENT/CONTROL VALUES FOR ALGAL GROWTH

Species	Mean treatment/control		
	10^{-5} M	10^{-4} M	10^{-3} M
<i>C. acerosum</i>	1.11	0.752	0.162
<i>E. californica</i>	1.02	0.859	0.140
<i>M. thomasiana</i>	1.12	0.873	0.113
<i>P. morum</i>	0.935	0.704	0.173
<i>S. grevilleana</i>	1.03	0.791	0.122

M juglone concentration ranged from 13 to 30%. Although *Eudorina* and *Micrasterias* were not significantly inhibited by a juglone concentration of 10^{-4} M in this study, continual exposure to juglone may result in an increased inhibitory effect. Rietveld (1983) suggested that allelopathic effects observed in the field are most likely the result of longer-term exposures to moderate juglone concentrations, such as 10^{-4} and 10^{-5} M. Thus, all species examined here may be inhibited to a greater degree by long-term exposure to juglone than is indicated by this study.

DISCUSSION

Ponder and Tadros (1985) reported that the concentration of juglone under black walnut ranged from 2.27×10^{-5} M within the top 8 cm of the soil and 0.9 m from the tree to 0 M at an increased distance and soil depth from the tree. This concentration would be adequate to cause previously described allelopathic inhibition in terrestrial systems (Rietveld, 1981). However, in aquatic systems, dilution of the compound delivered from the organism will occur. Thus, in order for allelopathy to occur in aquatic systems, additional mechanisms must exist for the accumulation of juglone to high enough concentrations to result in the allelopathic inhibition reported in this study and others such as those by Westfall et al. (1961) and Marking (1970).

Fisher (1978) reported that pines (*Pinus* spp.) often suppressed black walnut growth under dry soil conditions, while black walnut suppressed and often killed pine under poorly drained soil conditions. Fisher (1978) also showed that juglone was extractable and remained inhibitory for long periods from soil that was kept moist, while juglone of dry soil quickly disappeared and lost its inhibitory activity. It should be noted that the soil collected by Ponder and Tadros (1985) was dried for 24 hr before juglone was extracted. This may have considerably reduced the amount of juglone present within the samples.

Rietveld (1981) suggested that moist soil conditions limit the degradation of juglone. Therefore, it is likely that juglone will remain more stable and inhibitory in aquatic than terrestrial systems. This may allow an increased concentration to accumulate within aquatic systems. In addition, Ponder and Tadros (1985) sampled soil from a moderately well-drained site. Thus, a greater concentration of juglone than that measured by their study may be possible under an extremely wet moisture regime, such as in the soil near a lake and the lake water itself.

In addition to the build-up of juglone as a result of rainwash and runoff, fruit and leaf deposition within the lake will also occur. Juglone will become available from these by both leaching and decomposition within the sediment and hypolimnion. Phenolic compounds have been shown to accumulate in the

aquatic environment (Armstrong and Boalch, 1961; McLachlan and Cragie, 1964), and allelochemical accumulation was shown to occur within intertidal crevice sediments (Evans, 1986). Therefore, juglone may accumulate in the sediment of lakes located near black walnut. Little decomposition of juglone will likely occur in these sediments since juglone has been shown to inhibit bacteria and microorganisms (Shcherbanovskii et al., 1971; Sokolov et al., 1972; Didry et al., 1986).

Since spring turnover results in an abundance of available minerals and nutrients for algae, tremendous growth normally occurs. However, with turnover, the juglone accumulated within the sediment from the fall also will be dispersed. Should juglone concentrations be elevated at this time of year to levels shown by this study to inhibit algal growth, the annual algal production will be decreased substantially. This will result in reduced secondary production at all trophic levels.

Westfall et al. (1961) reported that fish were sedated, but not killed, by juglone concentrations between 6.2×10^{-6} and 1.3×10^{-5} M. Marking (1970) found that various fish were killed by concentrations ranging from 2.4×10^{-8} to 7.8×10^{-8} M juglone. The difference between results may have resulted in differences between the juglone obtained from extracts of black walnut by Westfall et al. (1961) and the juglone obtained from Aldrich Chemical Co. by Marking (1970). In either case, it appears that fish are more sensitive than algae to juglone present in the environment. Thus, should juglone be concentrated within the aquatic environment from 10^{-8} M to 10^{-5} M, fish toxicosis will occur in the absence of algal inhibition. An increase of algal biomass within the aquatic environment will result due to the removal of the higher trophic levels from the aquatic food web.

Intermediate concentrations of juglone, such as from 10^{-5} to 10^{-4} M, will be toxic to both fish and many algae species. However, some algae may persist, particularly those little affected by such juglone concentrations (i.e., *Micrasterias* and *Eudorina*). The species diversity of the lake under these conditions will be reduced, since it has been shown that most organisms are inhibited by juglone (Westfall et al., 1961; Marking, 1970). However, it is likely that an increase in algal biomass will occur at these intermediate concentrations, resulting from the fish toxicity. Should juglone accumulate to concentrations of 10^{-4} – 10^{-3} M, all organisms would be inhibited. Extreme reduction of both primary and secondary production within the lake environment will likely result.

It would be difficult to examine the effects caused specifically by the presence of juglone within the natural aquatic system. This is due to the occurrence of other interactions between organisms, i.e., competition and allelopathic interactions such as those described by Keating (1977, 1978). Thus, the initial step for the determination of the effects of juglone within aquatic systems is to analyze the effects of juglone upon a variety of organisms. Potential ecological

situations may then be deduced from the responses of aquatic organisms to various concentrations of juglone.

Additional work is required to locate the presence of juglone within the aquatic environment. A continuum of concentrations likely exists, depending upon such varying conditions as lake topography, nearby soil moisture levels, and number and size of nearby black walnut trees. An assay of juglone levels found within the natural environment would then allow for the correlation of laboratory and field observations. The effects of juglone within the aquatic environment may then be conclusively determined.

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APHID DETERRENCE BY GLUCOSE ESTERS IN GLANDULAR TRICHOME EXUDATE OF THE WILD TOMATO, *Lycopersicon pennellii*

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Abstract—Settling of the potato aphid, *Macrosiphum euphorbiae*, on feeding membranes was deterred by methanolic leaf rinses of *Lycopersicon pennellii*, or of its F₁ with tomato, *L. esculentum*. The active compounds in the *L. pennellii* rinsates were identified as 2,3,4-tri-*O*-acylglucoses bearing short to medium chain length fatty acids. These compounds are localized in the glandular exudate of the type IV trichomes and may accumulate to levels in excess of 400 $\mu\text{g}/\text{cm}^2$. In choice assays, purified glucose esters from *L. pennellii* reduced aphid settling at concentrations as low as 25 $\mu\text{g}/\text{cm}^2$; at concentrations of 150 $\mu\text{g}/\text{cm}^2$ or more, all aphids avoided treated areas. Glucose esters were also active in deterring aphid settling in no-choice assays. At 100 $\mu\text{g}/\text{cm}^2$, these esters resulted in increased levels of mortality after 48 hr.

Key Words—Aphid resistance, fatty acids, glucose ester, insect resistance, *Lycopersicon esculentum*, *Macrosiphum euphorbiae*, potato aphid, Homoptera, aphididae.

INTRODUCTION

Genetic diversity within the cultivated tomato has been seriously depleted by intensive selection pressure for agronomic traits. Wild germplasm is a potential source of resistance to 30 different diseases and 16 known arthropod pests of tomato (Rick, 1982). Currently, resistance to at least 15 different diseases has

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been incorporated into adapted cultivars utilizing this exotic germplasm (Rick, 1984). Incorporation of insect resistance into tomato has not been as successful because of the complex nature of resistance and a lack of understanding of its biochemical and genetic basis.

Lycopersicon pennellii Corr. (D'Arcy), a wild relative of the cultivated tomato, *L. esculentum* Mill, is resistant to many arthropod pests of tomato including the potato aphid, *Macrosiphum euphorbiae* Thomas; carmine spider mite, *Tetranychus cinnabarinus* Boisduval; two-spotted spider mite, *T. urticae* Koch; glasshouse whitefly, *Trialeurodes vaporariorum* Westw.; cotton bollworm, *Heliothis armigera* Hübner; and potato tuberworm, *Phthorimaea operculella*, Zell. (De Ponti *et al.*, 1975; Gentile and Stoner, 1968; Gentile *et al.*, 1968, 1969; Juvik *et al.*, 1982). These authors have suggested that the resistance in *L. pennellii* is due to the entrapment of these pests in the viscous glandular exudate of the type IV trichomes, which are not present on the cultivated tomato (Luckwill, 1943).

However, aphid resistance in *L. pennellii* cannot be completely attributed to entrapment in the sticky glandular exudate. Although potato aphid mortality in leaf cages is significantly higher on *L. pennellii* and its F₁ than on *L. esculentum*, few adult aphids become entrapped in the glandular exudate (Goffreda *et al.*, 1988). Aphid settling is dramatically reduced on *L. pennellii*, suggesting that starvation may contribute to the high aphid mortality.

We recently characterized aphid feeding behavior on *L. pennellii*, *L. esculentum*, and F₁ plants using electronic monitoring techniques (Goffreda *et al.*, 1988). Feeding behavior on *L. pennellii* and F₁ plants is characterized by an increase in preprobe time, a reduction in the number of probes per unit time, and a decrease in the total time spent probing, relative to tomato. The transfer of *L. pennellii*'s glandular exudate to *L. esculentum* results in feeding behavior resembling that on *L. pennellii* and F₁ plants. Conversely, removal of the type IV glandular exudate from *L. pennellii* and the F₁ with various solvents decreases preprobe time and increases the number of probes and total time spent probing. These results suggest that the chemistry of the glandular exudate of *L. pennellii* also deters aphid feeding.

Although previous work associated *L. pennellii*'s type IV trichome exudate with resistance to aphids and other insects, relatively little is known about the chemical basis of this resistance. In this study we show that application of *L. pennellii* type IV glandular exudate to artificial feeding surfaces deters aphid settling, and we identify the chemical constituents responsible for this activity.

METHODS AND MATERIALS

Plant and Aphid Culture. Plants of *L. esculentum* cv. New Yorker, *L. pennellii* (LA 716), and their F₁ were grown from seed in soilless media, a modification of Cornell mix (Boodley and Sheldrake, 1973), supplemented with

a slow-release fertilizer. Plants were not treated with pesticides. All plants used for chemical extraction were grown to maturity (*ca.* 3 months old) in a growth chamber maintained at 29°C day and 27°C night with a relative humidity of *ca.* 65%. The chamber was illuminated with *ca.* 1300 $\mu\text{E}/\text{m}^2/\text{s}$ of fluorescent and incandescent light for a 16-hr photophase.

Aphids were obtained from a colony established from a single apterous potato aphid (*Macrosiphum euphorbiae*) collected from tomato in Ithaca, New York, in September 1985 and maintained on tomato plants cv. New Yorker in a growth chamber at 20°C. The chamber was illuminated with diffuse fluorescent light of *ca.* 55 $\mu\text{E}/\text{m}^2/\text{s}$ intensity. Apteræ were gently shaken from the foliage and starved for *ca.* 3 hr prior to bioassays.

Aphid Settling Assays. Potato aphid settling behavior was assayed using a modification of the procedure described by Avé *et al.* (1987). Each feeding chamber was constructed by cutting two 1-cm² feeding ports in a hollow polyethylene stopper (Nalgene No. 7). A circular disk of unstretched Parafilm (American Can Company) was placed on top of the stopper and secured with another layer of Parafilm (Figure 1). A 0.9-ml volume of 20% sucrose was supported atop the feeding apparatus with another layer of stretched Parafilm, and yellow transparent disks of cellulose acetate were placed over each of the feeding ports. Tested compounds and extracts were applied to the exposed surface of the unstretched Parafilm, which was directly encountered by the aphids.

Choice Bioassays. A solution of the test material was applied to the surface of one of the two feeding ports, designated as the test port, and an equivalent volume of solvent was applied to the other feeding area (control port). Control feeding chambers were constructed by applying solvent alone to both the test and control feeding ports. Four aphids were placed in each chamber, and the distribution of aphids between the test and control ports was recorded at 2, 6, and 24 hr.

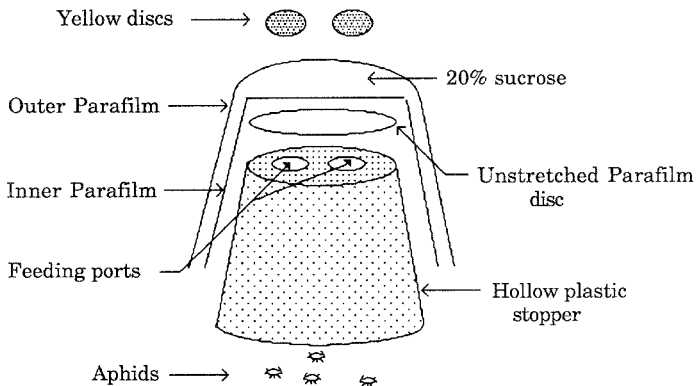


FIG. 1. Schematic diagram of aphid feeding chamber utilized in choice and no-choice assays.

Since aphids are gregarious (Dixon, 1973), each chamber was analyzed as a single experimental unit. Chambers in which there was either an equal aphid distribution between both ports or more aphids settled on the test port were scored as showing no response (NR). Chambers in which there were more aphids settled on the control port were scored as showing a response (R). Since chambers with equal aphid distributions were scored NR, the proportion of control chambers scored NR was expected to be greater than 50% on average. If a compound or extract strongly deters aphid settling, the proportion of chambers scored NR will be significantly lower than that of the control.

No-Choice Bioassays. A solution of the test material was applied to both feeding ports, and only a single aphid was placed in each chamber. Aphid settling was recorded at 2, 6, and 24 hr in short-term no-choice assays. In long-term no-choice studies each chamber was scored for whether or not the aphid was alive, and, for those still living, whether it was settled on a feeding port at 24, 48, and 72 hr.

Bioassay of Leaf Rinses. Since previous work (Goffreda *et al.*, 1988) has demonstrated that the feeding deterrent activity of *L. pennellii* can be removed by washing the leaf in alcohols, leaf rinses were assayed for activity in choice bioassays. Approximately 200 cm² of leaflets of *L. esculentum* cv. New Yorker, *L. pennellii* (LA 716), or their F₁ were briefly (1–3 sec) dipped in two 15-ml volumes of methanol (Fisher HPLC Grade). Microscopic examination of treated leaves revealed that the methanolic dip effectively removed the type IV trichome exudate droplets with no apparent damage to the membrane-enclosed type VI trichomes. A number of solvents are capable of efficient removal of type IV trichome exudate droplets. Alcohols such as methanol cause the least disruption of leaf and trichome metabolism; within 48 hr, leaflets treated with methanol regenerate exudate droplets. However, halogenated solvents cause wilting and death of the leaflet within a few hours after exposure.

The first and second rinses were combined and concentrated so that 10 μ l of this extract represented half the exudate obtained from 1 cm² of leaf surface. In choice bioassays, 10 μ l of the concentrated rinse was applied to the test port and allowed to dry as described above. There were 24 replicates per genotype arranged in a completely randomized design. Data were analyzed by partitioning of the likelihood ratio chi-square (*G* statistic) as described by Shaffer (1973).

Identification of Feeding Deterrents in L. pennellii Type IV Trichome Exudate. The glandular exudates of several hundred type IV trichomes were individually collected with a finely drawn capillary tube and injected into a Varian 3740 gas chromatograph equipped with a flame-ionization detector and a 25-m BP-5 fused silica narrow-bore capillary column (5% phenyl, methyl silicone, bonded phase, 0.25 μ m) (SGE, Australia). The injection port temperature was maintained at 260°C, and the column was held at 50°C for 5 min and programmed to 250°C at 4°C/min. Identity of fatty acids in the exudate was con-

firmed by comparison of retention times and by coinjection with both free and methyl esters of fatty acid standards. Methyl ester derivatives were prepared by treatment with BF_3 -methanol (Pierce Chemical Co). GC-MS of fatty acids was carried out on a Finnigan 3300 gas chromatograph-mass spectrometer in electron impact and chemical ionization modes.

Thin-layer chromatography (TLC) analysis of sugars in hydrolyzed and untreated trichome exudates was carried out on silica gel TLC plates (DC Fertigplatten Plate-Kieselgel 60 F-254) eluted twice with *n*-butanol, glacial acetic acid, ether, and distilled water (9:6:3:1) (Harborne, 1973). The plate was stained by heating with aniline-diphenylamine-phosphoric acid (Schwimmer and Bevenue, 1956).

Preparative isolation of *L. pennellii* glucose esters was achieved by a brief (1–3 sec) dip of 300 leaves in 1 liter of methanol to collect the polar epicuticular lipids; 12.5 ml of water was added to a 37.5-ml subsample of the leaf rinse and partitioned against hexane. The aqueous fraction was concentrated to a yellowish oil using a rotary evaporator equipped with a vacuum pump. The sample was taken up in a minimal volume of chloroform and purified over a Florisil column (30 × 2.5 cm) eluted with a 1.2-liter gradient of 100% chloroform to 50% chloroform-acetone. Glucose esters eluted as a clear viscous oil at approximately 20% acetone. Individual glucose esters were purified for characterization by reverse-phase HPLC (210 nm, Waters 5-micron C18 radial compression column). Authentic *L. pennellii* glucose ester standards were graciously provided by Dr. Basil Burke of Plant Cell Research Institute Inc. (Dublin, California).

Fatty acid compositions were identified as described above. Positions of esterification on the glucose esters were established by [^1H]NMR spectra recorded in CDCl_3 using a Varian XL-400 spectrometer. Spectra were referenced to CDCl_3 at $\delta 7.26$. High-resolution positive ion desorption chemical ionization MS spectra of purified glucose esters were recorded on a Kratos MS 890 spectrometer.

Bioassay of Purified Glucose Esters. Biological activity of purified glucose esters was assayed using choice and both short- and long-term no-choice assays as described. To ensure even coverage of glucose esters on the Parafilm, 2.5 μl of a solution of glucose esters dissolved in a mixture of acetone, chloroform, and paraffin oil (15:3:1) was applied onto the feeding membrane surface and allowed to dry. All treatments were arranged in a completely randomized design. In choice assays, glucose esters were tested at 0, 25, 50, 100, 150, and 200 $\mu\text{g}/\text{cm}^2$, with 22 replicates per concentration. In short-term no-choice assays, glucose esters were tested at 0, 50, 100, and 200 $\mu\text{g}/\text{cm}^2$, with 20 replicates per concentration. To determine if there was a relationship between the concentration of sugar esters applied and aphid settling, data were analyzed for homogeneity and tested for linearity by regression of the arcsine square root of the

proportion on the concentration of purified total glucose esters (Snedecor and Cochran, 1980). Long-term no-choice assays were conducted at two levels, 0 and 100 $\mu\text{g}/\text{cm}^2$, and analyzed by partitioning of the likelihood ratio chi square (Shaffer, 1973).

RESULTS

Leaf Rinse Bioassays. Leaf rinses from both *L. pennellii* and its F_1 with *L. esculentum* strongly deterred aphid settling on treated feeding ports in choice assays (Figure 2). Aphid response to the *L. esculentum* rinse did not differ significantly from the control chambers (Table 1). Aphid response to *L. pennellii* and F_1 leaf rinses also did not differ significantly. The chi square from the pooled data (*L. esculentum* and control vs. *L. pennellii* and F_1) was highly significant and accounted for over 90% of the total variation at each time period (Table 1).

Identification of Deterrents in Type IV Glandular Exudate and Leaf Rinses. TLC and HPLC analyses of both type IV trichome exudate and methanolic leaf rinses showed that the primary constituents of *L. pennellii* trichome exudate are glucose esters of branched short and medium chain length (C_4 to C_{12}) fatty acids. TLC analysis showed that *L. pennellii* leaf rinses, hydrolyzed by treatment with weak base, contained free glucose, which was not present in unhydrolyzed samples, suggesting esterification. Similarly, fatty acids were also not detected by GC unless injection port temperatures exceeded 150°C. The predominant fatty acids, identified by GC and GC-MS of the free acids and methyl

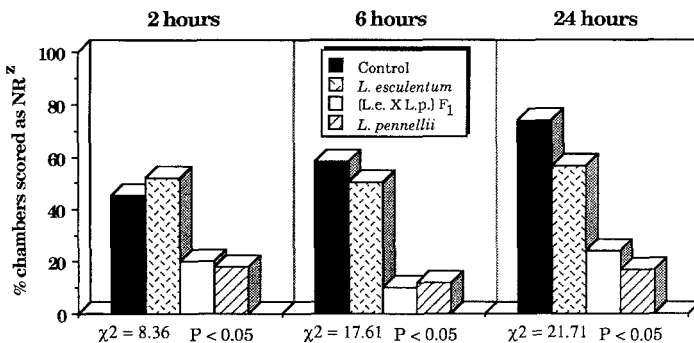


FIG. 2. Aphid settling response to leaf dip rinsate from *L. esculentum*, *L. pennellii*, and their F_1 hybrid in choice assays at 2, 6, and 24 hr. ²Expressed as percent of the chambers scored as NR. Chambers in which there was either an equal distribution of aphids between both ports or more aphids settled on the test port were scored as showing no response (NR).

TABLE 1. APHID SETTLING RESPONSE TO LEAF DIP RINSATES OF *L. pennellii* (L.p.), *L. esculentum* (L.e.), AND F₁ AS ANALYZED BY ORTHOGONAL PARTITIONING OF LIKELIHOOD RATIO CHI SQUARE FROM CHOICE STUDIES

Time (hr)	Likelihood ratio chi square ^a			
	Total (df = 3)	L.p. vs. F ₁ (df = 1)	Control (C) vs. L.e. (df = 1)	(L.p. and F ₁) vs. (C and L.e.) (df = 1)
2	8.36 ^b	0.03	0.22	8.11**
6	17.62***	0.03	0.26	17.33***
24	21.72***	0.36	1.55	19.81***

^aLikelihood ratio chi square was obtained from analysis of frequency tables of chambers scored as NR and R for the different treatments.

^b*, **, ***: $P < 0.05, 0.01, \text{ and } 0.001$, respectively.

esters, include 2-methylpropanoic, 3-methylbutanoic, 8-methylnonanoic, *n*-decanoic, and *n*-dodecanoic acids. GC analysis of individually collected, type IV droplets possessed high levels of these esterified fatty acids in the same proportions as they occur in the glucose esters from the leaf rinses. Individually collected type VI trichomes did not contain detectable quantities of the esterified fatty acids.

The [¹H]NMR spectrum of total glucose esters from *L. pennellii* showed, in agreement with Burke *et al.* (1987), that fatty acid esterification was at positions 2, 3, and 4 of glucose for all members of the complex: δ 4.92, *dd*, $J = 9.8, 3.7$ Hz; δ 5.69, *t*, $J = 9.8$ Hz; and δ 4.95, *t*, $J = 9.8$ Hz for H-2, H-3, and H-4, respectively, of the α anomer. As the glucose esters are hygroscopic, and in the field are constantly exposed to moisture, it is likely that the esters exist as an anomeric mixture both on the plant as well as *in vitro*. We were unable to assign precise positions of each fatty acid on individual glucose esters by either NMR or MS. High-resolution MS of a purified ester identified by GC as composed of di-2-methylpropanoic, *n*-decanoic fatty acids gave a mass of 457.2799, satisfying the formula for C₂₄H₄₁O₈ (M + H - H₂O; calc. 457.2801).

Activity of L. pennellii Total Glucose Esters. In choice assays, there were highly significant deviations ($P < 0.001$) in the proportion of chambers scored as showing no response (NR) at different levels of glucose ester application for the 2-, 6-, and 24-hr time periods (Table 2). The test for linear trend was also highly significant at each time period ($P < 0.001$), indicating that there was an increase in aphid deterrence with increasing levels of glucose ester application. Aphids completely avoided the test port at concentrations of 150 $\mu\text{g}/\text{cm}^2$ and higher.

TABLE 2. APHID SETTling RESPONSE TO *L. pennellii* TOTAL GLUCOSE ESTERS IN CHOICE ASSAYS^a

Concentration ($\mu\text{g}/\text{cm}^2$)	Time					
	2 hours		6 hours		24 hours	
	NR (%)	<i>N</i>	NR (%)	<i>N</i>	NR (%)	<i>N</i>
Control (0)	70	20	50	20	55	20
25	44	18	44	18	21	19
50	17	18	12	16	32	19
100	16	19	9	22	15	20
150	0	19	0	19	0	21
200	0	21	0	21	0	19
Homogeneity χ^2	40.78 ($P < 0.001$)		31.33 ($P < 0.001$)		26.88 ($P < 0.001$)	
Test for linear trend ($b = 0$)	$P < 0.001$		$P < 0.001$		$P < 0.001$	

^a Expressed as percent of chambers scored as exhibiting no response (NR).

^b Test for linear trend in proportions is calculated by regression of the arcsine square root of the proportion NR on the concentration of purified total glucose ester applied to the feeding membrane.

In short-term no-choice assays, there was a highly significant shift ($P < 0.005$) in the proportion of aphids settled at each time period (Figure 3). There was also a highly significant linear trend ($P < 0.001$) in aphid settling with increasing levels of glucose ester application to the feeding areas. In long-term

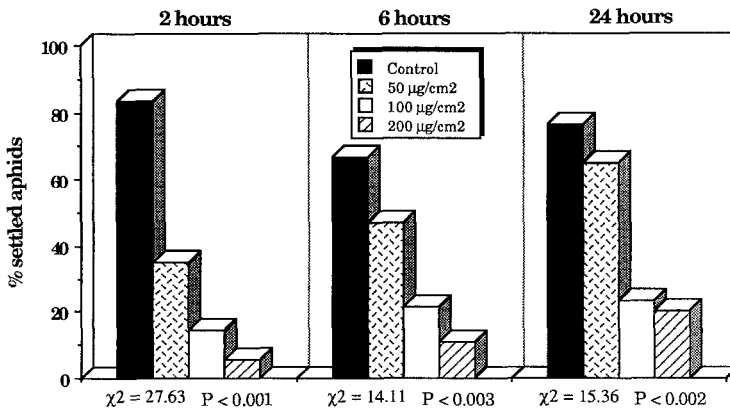


FIG. 3. Aphid settling response to *L. pennellii* total glucose esters in no-choice assays at 2, 6, and 24 hr.

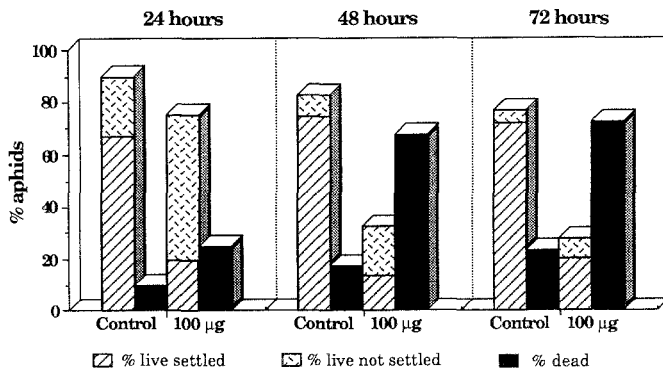


FIG. 4. Effect of *L. pennellii* total glucose esters ($100 \mu\text{g}/\text{cm}^2$) on aphid settling and mortality in long-term no-choice assays.

TABLE 3. ORTHOGONAL PARTITIONING OF LIKELIHOOD RATIO CHI SQUARE CALCULATED FROM APHID SETTLING AND MORTALITY DATA IN LONG-TERM NO-CHOICE STUDIES ON EFFECT OF TOTAL GLUCOSE ESTERS ($100 \mu\text{g}/\text{cm}^2$)

Time (hr)	Likelihood ratio chi square ^a		
	Total (df = 2)	Dead vs. alive (df = 1)	Settled vs. not settled (df = 1)
24	19.17*** ^b	3.20	15.97***
48	31.67***	21.55***	10.12***
72	23.03***	20.21***	2.82

^aLikelihood ratio chi square was obtained from analysis of frequency tables of chambers scored as dead, settled, and not settled for the $100 \mu\text{g}/\text{cm}^2$ and control treatments.

^b***, $P < 0.001$.

no-choice assays, the proportion of living aphids settled at 24 and 48 hr was significantly lower in chambers in which $100 \mu\text{g}/\text{cm}^2$ of total glucose esters were applied to the feeding area than in control chambers (Figure 4; Table 3). At 48 and 72 hr, the proportion of dead aphids in treated chambers was significantly higher than in control chambers ($P < 0.001$).

DISCUSSION

Settling of the potato aphid, *Macrosiphum euphorbiae*, is deterred in artificial feeding chambers by methanolic leaf rinses of *L. pennellii* and its F_1 with *L. esculentum*. In contrast, *L. esculentum* methanolic leaf rinses have no effect on aphid settling behavior.

Active compounds in the *L. pennellii* leaf rinses are 2,3,4-tri-*O*-acyl glucoses comprised of short to medium chain length fatty acids (C₄ to C₁₂). Individual collection of type IV trichome exudates confirms that these compounds are localized in the glandular exudate of this trichome. Sugar esters of short and medium chain length fatty acids are the predominant compounds in the leaf rinses of *L. pennellii* and the F₁. Few other components are detectable in either the leaf rinses or the type IV trichome exudate of these plants.

Our structural identification of these glucose esters in the trichome exudate of *L. pennellii* is in agreement with that published by Burke *et al.* (1987) and with the properties of authentic *L. pennellii* glucose ester standards (kindly supplied by B.A. Burke). Sucrose esters of similar fatty acids have been isolated from the trichomes of other insect-resistant species within the family Solanaceae, including *Nicotiana tabacum*, *Solanum berthaultii*, and *Lycopersicon hirsutum* (Severson *et al.*, 1985; King *et al.*, 1986, 1987).

Insecticidal properties of free decanoic and dodecanoic acids and their derivatives have been known for over 50 years (Siegler and Popenoe, 1925; Shepard, 1951). The biological effects of endogenous fatty acid esters have only recently been studied within the family Solanaceae. Johnson and Severson (1984) characterized the leaf surface chemistry of nearly 30 tobacco accessions and found one accession with high sucrose ester levels and low docosanol levels that was resistant to damage from the tobacco budworm, *Heliothis virescens*. Other research indicates that 3-methylvalerate-substituted sucrose esters of tobacco also inhibit gram-positive bacteria, including *Bacillus subtilis*, *B. cereus*, and *Mycobacterium thermosphaerium* (Cutler *et al.*, 1986). Recent work with *Solanum berthaultii* also implicates the involvement of sucrose esters in this species' resistance to aphids (Neal *et al.*, in press) and potato late blight, *Phytophthora infestans* (Holley *et al.*, 1987).

L. pennellii glucose esters are perceived by aphids at concentrations of 25 µg/cm² in choice bioassays (Table 2). It is unlikely that the aphid is responding to the viscous properties of the glucose ester since these compounds only form a microscopic film at this low concentration. It is possible that abiotic or biotic conditions may hydrolyze the ester linkage, thereby liberating free short and medium chain fatty acids. Fatty acids with chain lengths of C₉ to C₁₃ are disruptive to settling of the green peach aphid, *Myzus persicae* Sulzer, at concentrations of 1–100 µg/cm² (Greenway *et al.*, 1978). In our assays, *n*-decanoic or *n*-dodecanoic acids repel potato aphid settling at concentration of 50 µg/cm² (unpublished data).

L. pennellii glucose esters are also active in no-choice assays, although the threshold level of activity is higher than in choice assays; between 50 and 100 µg/cm² was required to detect a deterrent response at 24 hr (Figure 3). In long-term no-choice assays, there was significantly higher mortality in chambers treated with 100 µg/cm² than control chambers at 48 hr and 72 hr, probably

because the aphids died from starvation since they would not settle and feed (Table 3; Figure 4). There was no evidence of aphid habituation to glucose esters because the proportion of aphids that were settled on the treated feeding area remained constant through the course of the experiment. This high level of aphid deterrence possessed by glucose esters may be of practical significance in reducing the rate of virus transmission because, in many cases, aphids cease to be viruliferous minutes after acquiring a nonpersistent virus (Bradley, 1959; Harris, 1977).

The activity of individual glucose esters is not different from that of the total glucose esters, suggesting the absence of either synergistic or antagonistic interaction between individual glucose esters. Furthermore, there was little variation in deterrent activity among individual glucose ester fractions, suggesting that fatty acid chain length is not critical for deterrence (data not shown).

Polar epicuticular lipids can accumulate to concentrations exceeding $400 \mu\text{g}/\text{cm}^2$ on *L. pennellii* foliage (Fobes *et al.*, 1985). Data presented here suggest that glucose esters may be active deterrents of aphid settling at concentrations far lower than those reported on mature *L. pennellii* plants: total avoidance was observed at concentrations as low as $150 \mu\text{g}/\text{cm}^2$.

This high level of deterrent activity of glucose esters at relatively low concentrations should facilitate the transfer of aphid resistance into the cultivated tomato in an applied breeding program. Our research has determined that the presence of the type IV trichome is relatively simply inherited (Lemke and Mutschler, 1984), but we have had difficulty in retaining high densities of type IV trichomes bearing large exudate droplets in hybrid populations. Since fatty acid chain length on *L. pennellii* glucose esters may not be critical to the activity of these compounds in choice assays, screening plants for total epicuticular glucose esters should be effective in the development of resistant hybrids. We have developed a rapid colorimetric assay to evaluate sugar ester accumulation in large segregating populations. We are in the process of evaluating this assay as a selection technique for the development of insect-resistant tomato cultivars.

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ALLELOPATHIC RESEARCH OF SUBTROPICAL
VEGETATION IN TAIWAN. IV.
Comparative Phytotoxic Nature of Leachate from Four
Subtropical Grasses^{1,2}

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Abstract—The phytotoxicity of plant leachates was evaluated from four subtropical grasses: *Brachiaria mutica*, *Digitaria decumbens*, *Imperata cylindrica* var. Major, and *Panicum repens*. The aqueous leachate of each grass was used to water the growth of the four grasses in pots. The leachates exhibited variable inhibition of grass growth as compared to the tap water control. By the 41st day after treatment, the leachate of *D. decumbens* significantly suppressed the growth of itself and retarded that of *B. mutica* and *P. repens*. The growth of *B. mutica* was inhibited by its own leachate, but that of *I. cylindrica* was not affected by any of the grass leachates. In crop growth rate (CGR) analysis, the four grass leachates exhibited a similar inhibition pattern. In laboratory bioassays, the leachates showed a significant phytotoxic effect on the radicle growth of ryegrass and lettuce. Six phytotoxic phenolics were quantitatively compared by high-performance liquid chromatography, and the amount of compounds varied with species. The highest total amount of phytotoxic phenolics occurred in *D. decumbens*, followed, in decreasing order, by *P. repens*, *B. mutica*, and *I. cylindrica*. These findings show that the leachates of four grasses possess phytotoxic compounds that may play a significant role in grass dominance in the field.

Key Words—Allelopathy, phytotoxicity, phytotoxic phenolics, grass leachate, *Digitaria decumbens*, *Brachiaria mutica*, *Panicum repens*, *Imperata cylindrica*.

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INTRODUCTION

Organic and inorganic substances are commonly leached out from the above-ground plant parts by rainfall, mist, fog, and dew through ecological processes (Tukey, 1969, 1970, 1971; del Moral and Muller, 1969; Chou and Muller, 1972). This phenomenon was described by Stephen Hales and de Saussure (see Tukey, 1971) in the early 18th century, and subsequent studies were conducted in many parts of the world (Tukey, 1970). Phytotoxic metabolites in leachates often play an important role in the regulation of plant dominance, succession, stability of plant communities, and agricultural productivity (Muller, 1969; Chou, 1987; Rice, 1984). The leaching phenomenon in the humid region of Taiwan is of particular importance because of the substantial amount of annual precipitation there. Chou and coworkers studied the ecological functions of plant leachates in various vegetations (Chou, 1975; Chou and Chen, 1976) and found that some leachates exhibited allelopathic properties. Chou and Young (1975) evaluated the phytotoxicity of 12 subtropical grasses and found at least seven phytotoxic substances present in the aqueous extracts of the grasses. This led us to further investigate the phytotoxic nature of leachates from four subtropical grasses, *Brachiaria mutica*, *Digitaria decumbens*, *Imperata cylindrica*, and *Panicum repens*, in order to clarify biochemical interference both intra- and interspecifically.

METHODS AND MATERIALS

Four subtropical grasses, *Brachiaria mutica*, *Digitaria decumbens*, *Imperata cylindrica* and *Panicum repens*, were selected for study. Plant material of *B. mutica* and *I. cylindrica* was collected at the farm of the Institute of Botany, Academia Sinica, Taipei, and the other two species were obtained from the Hsinchu Experiment Station of the Taiwan Livestock Research Institute. Fresh grass leaves were collected and allowed to air dry. The soil used for pot experiments was obtained from the field of the Institute of Botany, Academia Sinica, and the visible plant residues were removed before placing into the pots.

Each grass leachate was obtained by using a leaching apparatus (Figure 1), consisting of a steel frame with three layers (A, B, and C). Layer A was a 55 × 40 × 15-cm plastic tray with numerous needle-sized holes (2 cm between holes), which was filled with 3 liters of tap water. The tap water dripped through the holes as an artificial rain and 1 kg of chopped leaves of each grass was placed on a similar tray of layer B. The leachate from layer B was collected in layer C. About 800 ml of each leachate was collected daily, and the leachate was returned to tray A and recirculated for another 48 hr. The leachate was then stored in a cold room at 5°C prior to analysis. The grass was replaced by fresh

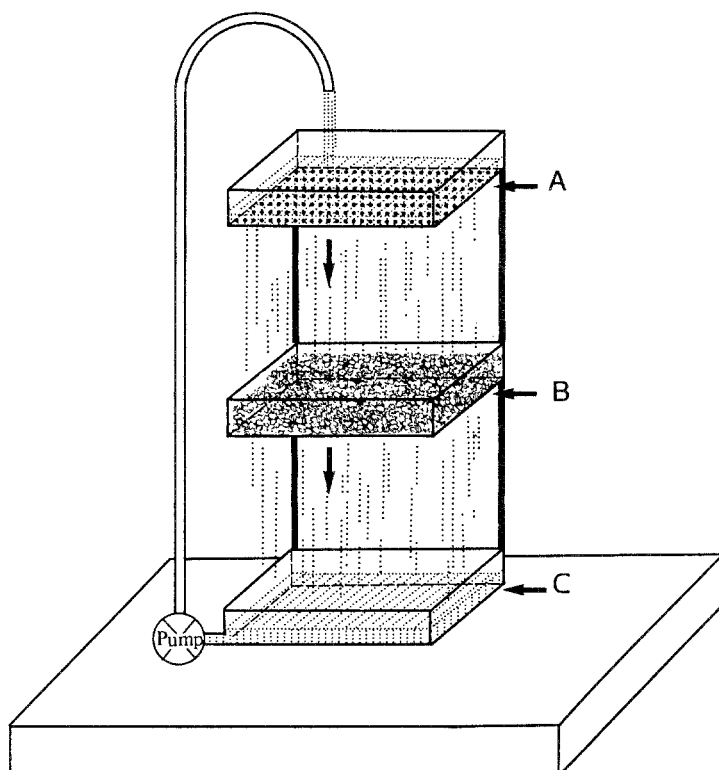


FIG. 1. The leaching apparatus system: A = a plastic tray, $55 \times 40 \times 15$ cm with numerous needle-sized holes (2 cm between holes) was filled with tap water, which dripped through the holes to make an artificial raindrip; B = the same as tray A was filled with chopped plant material to receive the artificial raindrip from tray A; C = a plastic tray, like that of A and B, without holes to receive plant leachate from tray B. The pump recirculated the water from tray C to tray A.

material every week. Of the 800-ml leachate, 200 ml was filtered through a Whatman No. 42 filter paper and used for bioassay or chemical analyses. The remaining leachate was used for watering the grasses grown in pots in the greenhouse of the Academia Sinica, Taipei.

Laboratory Analyses. The grass leachate was bioassayed to determine phytotoxicity (Chou and Young, 1975). Seeds of lettuce (*Lactuca sativa* var. Great Lakes 366) and ryegrass (*Lolium multiflorum*) were used as test species. Distilled water served as the control. Each bioassay included three replications and was incubated at 25°C for 72 hr. After incubation, the radicle length of each seedling was measured and the phytotoxicity expressed as percent inhibition as compared with the control.

The cation content of each leachate was determined by an atomic absorption spectrophotometer (Perkin-Elmer, model 300) following the techniques described in Analytical Methods for Absorption Spectrophotometer (Perkin-Elmer, 1971).

The techniques for isolating phytotoxic substances present in the leachates were described by Chou and Young (1975). The identification of phytotoxic substances was performed mainly by means of paper chromatography using two solvent systems and four detecting methods (Chou and Lin, 1976). The amount of phytotoxic phenolics present in each grass leachate was determined by a high-performance liquid chromatograph (HPLC) (LDC model 1203) and co-chromatographed by using authentic phenolics for comparison (Chou and Kuo, 1986).

Pot Experiment. Each pot, 25 × 30 cm size, was filled with 10kg fresh soil and then planted with 15-cm long stolons or rhizomes, with four replications. The pots were placed in a greenhouse at Nankang and were irrigated with tap water until the grass grew healthily. The pot was then irrigated with the grass leachate obtained from laboratory; a similar pot was irrigated with tap water as a control. The length of the main leaf of the grasses was measured on two successive dates, days 11 and 41 after irrigation, to detect the effect of leachates. In addition, on days 59 and 119 after irrigation, the aboveground part of the grasses was harvested and the dry weight measured. The crop growth rate (CGR) of each grass in every treatment was compared in order to see the effect of leachates on the grass growth. The crop growth rate is defined as follows:

$$\text{CGR} = \frac{\text{Difference of biomass in weight between two measured times (g)}}{\text{Length of time between two measured times (day)}}$$

RESULTS

Effects of Grass Leachates on Grass Growth. The growth of grasses in pots was measured 11 and 41 days after watering with leachate or tap water to see the effects of each grass leachate on grass growth. Results of the leaf length measurement are shown in Table 1, which indicate that the grass growth, at the early stage, was noticeably suppressed by leachates compared to tap water. The leachates obtained from *Digitaria decumbens*, *Brachiaria mutica*, and *Panicum repens* inhibited leaf growth of *D. decumbens*. At the 41st day after treatment, the leachate of *D. decumbens* inhibited its own growth and also suppressed the growth of *B. mutica* and *P. repens*. The leachate from *B. mutica* was also autotoxic. However, the growth of *Imperata cylindrica* was not significantly affected by the leachates.

The crop growth rate (CGR) was calculated based on the dry weight of

TABLE 1. EFFECTS OF GRASS LEACHATES ON GROWTH OF FOUR GRASSES^a

Species of leachate donor	DAI ^b	Species of leachate receiver													
		<i>D. decumbens</i>				<i>B. mutica</i>				<i>P. repens</i>				<i>I. cylindrica</i>	
		Length (cm)	% of control	Length (cm)	% of control	Length (cm)	% of control	Length (cm)	% of control	Length (cm)	% of control	Length (cm)	% of control		
Tap water (control)	11	47.2	100	61.7	100	42.2	100	33.3	100	72.9	100	33.3	100		
	41	160.7	100	138.4	100	88.7	100	88.7	100	88.7	100	72.9	100		
<i>D. decumbens</i>	11	27.8*	59	42.6*	69	31.0*	73	30.4	73	75.7	82	30.4	91		
	41	114.2**	71	114.5**	83	73.1**	82	75.7	82	75.7	82	75.7	104		
<i>B. mutica</i>	11	37.8**	80	40.1*	65	34.2**	81	30.6	81	77.2	96	30.6	92		
	41	156.6	97	114.4	81	85.4	96	77.2	96	77.2	96	77.2	99		
<i>P. repens</i>	11	43.5**	92	48.5**	79	33.8	80	30.8	80	58.4	92	30.8	93		
	41	153.3	95	168.2	122	81.8	92	58.4	92	58.4	92	58.4	80		
<i>I. cylindrica</i>	11	37.0**	78	48.1**	78	36.3	86	30.6	86	69.6	102	30.6	92		
	41	162.6	101	123.9	90	90.3	102	69.6	102	69.6	102	69.6	96		

^aData were obtained from the average of four replications of the aboveground plant parts on two measuring dates.

^bDAI: days after irrigation.

^cStatistical significance at * $P < 0.01$ and ** $P < 0.05$ level, respectively, as compared with tap water control by using a Student *t* test.

grasses harvested at days 59 and 119 following irrigation with leachates (Table 2). The leachate of *D. decumbens* significantly suppressed the growth of itself and that of *B. mutica* in both cuttings but stimulated the growth of *P. repens* and *I. cylindrica* at the second cutting. The leachate of *B. mutica* exhibited a pattern similar to that of *D. decumbens*, but the inhibition of the former was significantly lower than that of the latter. The leachate of *P. repens* was toxic to the growth of *B. mutica* in both cuttings and to that of *I. cylindrica* at the second cutting. Finally, the leachate of *I. cylindrica* suppressed the growth of *D. decumbens*, but stimulated the growth of *P. repens*. In conclusion, *D. decumbens* and *B. mutica* exhibited relatively high phytotoxic effects on the growth of themselves and appeared to be an autointoxicating phenomenon. However, *P. repens* was shown to be tolerant to the leachates of the aforementioned species.

Comparison of Phytotoxic Effects of Grass Leachates on Lettuce and Ryegrass. To further confirm the phytotoxic effect of the leachates on plant growth, bioassays were conducted using lettuce and ryegrass as test species. Leachates from *Digitaria decumbens*, *Brachiaria mutica*, and *Panicum repens* inhibited radicle growth of lettuce but had no effect on ryegrass (Table 3). Among them, the leachate of *D. decumbens* showed the highest phytotoxicity, which agreed with the findings in Table 2. The phytotoxicity of leachate obtained at different times of leaching was not significantly different, suggesting that the phytotoxic substances might continuously leach out within two weeks.

Identity of Phytotoxic Phenolics in Grass Leachate. By paper chromatography, six phytotoxic phenolics were identified. They include ferulic, *p*-coumaric, 2,4-dihydroxybenzoic, vanillic, *p*-hydroxybenzoic, and *p*-hydroxyphenylacetic acids, and three unidentified compounds. Of the three unknowns, one is a flavonoid and two are phenolic acids (Table 4). In addition, by using a HPLC analysis, the quantity of each of the identified phenolics was compared. The amount of phenolics present in the leachate of *D. decumbens* was the highest, *P. repens* was next, and *I. cylindrica* was the least (Table 4). The findings agreed with the bioassay results, in which the leachate from *D. decumbens* was the most phytotoxic.

Cation Contents of Leachates. The leachates were analyzed for cations by an atomic absorption spectrophotometer. The data were the average of cation contents from at least 30 leachates. The quantity of Cu, Zn, and Fe was as low as 0.3 ppm, and the content ranged from 39 to 170 ppm for K, 32 to 63 ppm for Na, 15 to 18 ppm for Ca, and 22 to 28 for Mg. Only the amounts of K, Na, and Mn were significantly different between species, but those of the remaining cations were not significantly different. Therefore, the cations present in the leachates of the grasses may not play a significant role in their growth performance.

pH and Osmotic Concentration of Grass Leachates. The pH of grass leachates ranged from 7.19 to 7.65. The osmotic concentrations were as low as 17.4,

TABLE 2. EFFECTS OF GRASS LEACHATES ON CROP GROWTH RATE (CGR) OF FOUR GRASSES^d

Species of leachate donor	Species of leachate receiver													
	<i>D. decumbens</i>				<i>B. mutica</i>				<i>P. repens</i>				<i>I. cylindrica</i>	
	DAI ^b	CGR	% of control		CGR	% of control			CGR	% of control			CGR	% of control
Tap water (control)	59	0.096	100		0.072	100			0.047	100			0.025	100
	119	0.204	100		0.215	100			0.127	100			0.105	100
<i>D. decumbens</i>	59	0.057* ^c	59		0.051*	71			0.045	96			0.024	93
	119	0.136*	67		0.149*	69			0.144	113			0.142*	135
<i>B. mutica</i>	59	0.072*	75		0.055*	76			0.059*	126			0.020**	80
	119	0.155*	76		0.150*	70			0.129	102			0.132*	125
<i>P. repens</i>	59	0.087	91		0.059**	82			0.049	104			0.012*	49
	119	0.191	94		0.164*	76			0.137	108			0.132	125
<i>I. cylindrica</i>	59	0.070*	74		0.074	102			0.080*	170			0.028	109
	119	0.158**	78		0.156*	73			0.136	107			0.116	110

^aThe definition of CGR is the difference of dry weight between two measured times per length of time measured and is expressed as dry weight/day.

^bDAI: days after irrigation.

^cStatistical significance at * $P < 0.01$ and ** $P < 0.05$ level, respectively, as compared with the tap water control by using a Student *t*-test.

TABLE 3. PHYTOTOXIC EFFECTS OF GRASS LEACHATES ON RADICLE GROWTH OF LETTUCE AND RYEGRASS^a

Source of leachate	Phytotoxicity (% inhibition)	
	Lettuce	Ryegrass
<i>Digitaria decumbens</i>	34.2* ^b	11.7
<i>Brachiaria mutica</i>	28.7*	9.9
<i>Panicum repens</i>	32.1*	15.0**
<i>Imperata cylindrica</i>	14.7	11.7

^aData are expressed as percent inhibition of growth on leachate as compared with a distilled water control.

^bStatistical significance at * $P < 0.01$ and ** $P < 0.05$ level, respectively, as compared with the distilled water control.

TABLE 4. QUANTITATIVE COMPARISON OF PHYTOTOXIC PHENOLICS IN FOUR GRASSES

Compound identified	Content (10^{-7} mol/g sample)			
	<i>I. cylindrica</i>	<i>D. decumbens</i>	<i>P. repens</i>	<i>B. mutica</i>
Ferulic acid		1.00	0.86	0.53
<i>P</i> -Coumaric acid	0.3	2.50	1.00	0.30
2,4-Dihydroxybenzoic acid		0.25	0.50	0.25
Vanillic acid		1.00	1.30	0.67
<i>p</i> -Hydroxybenzoic acid	0.5	0.50	0.50	0.50
<i>p</i> -Hydroxyphenyl acetic acid		2.50	2.50	0.75
Subtotal amount	0.8	7.75	6.66	3.00

at which concentration or below the plant growth would not be suppressed (Chou and Young, 1974). Thus, the previous findings of plant growth inhibition by the grass leachates were evidently due to phytotoxic inhibition rather than osmotic effect.

DISCUSSION

In previous studies, Chou and Young (1975) identified seven phytotoxins present in the aqueous extract of 12 subtropical grasses, and among these species *Digitaria decumbens* was the most phytotoxic to test plants. Moreover,

Chou (1977) presented further evidence that potentially phytotoxic substances were present in the aqueous fraction of the grass extracts. These unidentified phytotoxic compounds were tentatively identified as nitrogenous compounds of low molecular weight. In this study a phytotoxic flavonoid was found but the amount of the isolate was too small for structural identification. The physiological function and inhibition model on seed germination of 15 phytotoxins, including the aforementioned six phenolics, were studied, and findings will be presented in a subsequent report, which will interpret the role of phytotoxins in biochemical inhibition among plants. Compounds produced during the decomposition of grass residues in soil were also phytotoxic to the radicle growth of several test species (data are not shown). However, under natural conditions, the interactions among the aforementioned grasses have not been clearly demonstrated. The grass leachates obtained by the present leaching apparatus could not be the same but were very similar to those obtained in the field. One may argue that recirculated leachates do not occur in nature, and the concentration of the artificial grass leachate was also not the same as in the field. The author makes no argument about this; however, the present findings provide the evidence that under comparable conditions the grass leachate of *D. decumbens* clearly showed phytotoxicity. Since the productivity of *D. decumbens* declined several years after planting, Liang et al. (1983) found that the decline was due in part to biochemical inhibition of the grass. Thus, the present study is an attempt to answer the question by using grass leachate to test the growth of grasses inter- and intraspecifically. Because the pH and osmotic concentration were such that they did not affect plant growth (Chou and Young, 1974), the inhibition was thus surely due to the phytotoxic effect. In conclusion, the phytotoxic mechanism, therefore, plays an appreciable role in the formation of dominance and in the decline of productivity of *D. decumbens* in the field. The decline was overcome by a pangola grass-watermelon-pangola grass rotation management.

Black et al. (1969) proposed that the mechanism of plant dominance could be due in part to biochemical competition, in that some plants possess high efficiency in photosynthesis resulting in better growth. This phenomenon is particularly pronounced in C_4 plants, such as *D. decumbens*, which exhibit highly efficient photosynthesis, higher biomass production, and are aggressive against associated weeds grown nearby. According to the data of crop growth rate (CGR), the rate is higher in the *D. decumbens* than in the other three grasses (data were not shown). Chou and Lee (1987) found that the more nitrogen fertilizer was applied to the grass, the greater was the quantity of phytotoxins produced; this suggests that the increased growth of *D. decumbens* cultivars may produce more phytotoxins and may suppress the growth of competitors such as *P. repens* and *I. cylindrica*. Whittaker and Feeny (1971) and Putnam and Duke (1974) suggested that allelopathy could be used in agriculture by

breeders who can select cultivars that produce natural phytotoxins and thus lessen the use of herbicides and environmental pollution. Chou et al. (1987) have demonstrated such a case study by using kikuyu grass as a cover grass in deforested land, in which kikuyu suppresses the growth of weeds but not the growth of *Cunninghamia lanceolata*, a coniferous plant.

The amount of phytotoxins present in the aqueous leachate of *I. cylindrica* var. Major was the lowest of the species tested. Eussen and Niemann (1981) had identified eight phytotoxic phenolics, *p*- and *o*-coumaric, gentisic, vanillic, benzoic, and *p*-hydroxybenzoic acids, vanillin, and *p*-hydroxybenzaldehyde, from leaves of *I. cylindrica* (L.) Beauv. Of them, *o*-coumaric, gentisic, and benzoic acids, *p*-hydroxybenzaldehyde, and vanillin were not found in the leachate of *I. cylindrica* in the present study, due primarily to the difference of leachate and extract: the leachate contains less phytotoxin. The correlation between the phytotoxins and habitats and the real mechanism of interaction between the dominant and inferior grasses in the field still remain unclear.

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CHEMICAL CONSTITUENTS OF *Erysimum cheiranthoides* DETECTING OVIPOSITION BY THE CABBAGE BUTTERFLY, *Pieris rapae*

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Abstract—Avoidance of *Erysimum cheiranthoides* for oviposition by *Pieris rapae* has been attributed to the presence of water-soluble deterrents. The active material was extracted into *n*-butanol and isolated by a series of HPLC separations. TLC of the active fraction and visualization of individual constituents with Kedde's reagent indicated that cardenolides are responsible for deterring oviposition. UV spectra were also characteristic of cardenolides. Bioassays of selected known cardenolides revealed a general lack of activity, except for cymarins, which was as strongly deterrent as the most prominent cardenolide isolated in pure form from *E. cheiranthoides*. The results suggest that cardenolides in this plant can explain its escape from cabbage butterflies, but specific structural features of the glycosides are necessary for oviposition-deterrence activity.

Key Words—Cabbage butterfly, *Pieris rapae*, Lepidoptera, Pieridae, *Erysimum*, oviposition deterrent, cardenolides.

INTRODUCTION

The cabbage butterfly, *Pieris rapae* L., oviposits primarily on members of the Cruciferae and a few related plant families that are linked by the presence of glucosinolates (Verschaffelt, 1911; Ma and Schoonhoven, 1973); however, some crucifers are rejected by gravid butterflies and may be unsuitable for feeding by the larvae (Feeny, 1977; Rodman and Chew, 1980). These plants may contain specific groups of chemicals that offer a second line of defense against herbivores and other invading organisms (Feeny, 1977; Nielsen, 1978). However, representative cardenolides, cucurbitacins, and alkaloids, which have been

suggested as defensive compounds, failed to affect feeding and development of *P. rapae* larvae in the laboratory (Usher and Feeny, 1983). Nevertheless, the involvement of chemical constituents in deterring oviposition on nonhost plants has been clearly demonstrated. Polar extracts of the crucifers, *Erysimum cheiranthoides* L. and *Capsella bursa-pastoris* (L.), were particularly effective (Renwick and Radke, 1985). Further work on these unacceptable crucifers has shown that *E. cheiranthoides* contains oviposition stimulants as well as deterrents (Renwick and Radke, 1987), so the deterrents must be potent enough to outweigh the effects of the stimulant(s).

Despite the known presence of oviposition deterrents in nonhost plants, specific chemicals responsible for rejection of these plants have not been identified. In this report, we describe the systematic isolation and partial characterization of active compounds in *E. cheiranthoides* that deter oviposition by *P. rapae*.

METHODS AND MATERIALS

Insects. Butterflies used in bioassays were from a laboratory colony maintained on cabbage plants at ca. 22°C under fluorescent lights providing a photoperiod of 16 : 8 hours light-dark. The colony was originally started from field-collected butterflies and had completed 20–30 generations in the laboratory. No change in discriminatory behavior had been noted in this time. Pupae were separated by sex (Richards, 1940) and kept in screen cylinders (15 cm × 15 cm diam.) closed at the top and bottom with plastic Petri dishes. After eclosion, five pairs of butterflies were transferred to each bioassay cage in the greenhouse. Each cage was supplied with a vial of 10% sucrose solution containing yellow food coloring and a cotton wick to facilitate feeding.

Plants. *Erysimum cheiranthoides* plants were grown in artificial soil mix (Cornell Mix A) (Boodley and Sheldrake, 1977) from seeds originally collected by Dr. Frances S. Chew in Vermont. Supplemental lighting in the greenhouse was supplied by 400-W multivapor, high-intensity-discharge lamps, and the temperature was maintained at ca. 25°C. Foliage was harvested after four to five weeks, just before flowering began. Cabbage plants, *Brassica oleraceae* (var. Golden Acre), grown under the same conditions in 10-cm-diam. pots, reached a suitable size for bioassays after about six weeks.

Bioassays. Oviposition assays for deterrent activity were conducted in screen cages (48 × 48 × 48 cm) in a greenhouse, with supplemental lighting from 400-W multivapor lamps. For each replication, one treated and one control cabbage plant were placed in opposite corners of each cage for a period of 5 hr from 10:00 AM to 3:00 PM, and the eggs laid on each plant were counted. The positions of control and treated plants were alternated between cages to

eliminate possible positional effects. Dead butterflies were replaced daily to maintain five pairs in each cage. Extracts of fractions to be tested were applied to test plants in methanol or water using a Freon-propelled chromatographic sprayer. Both upper and lower leaf surfaces were sprayed with a total of 7 ml of test solution, and control plants were sprayed with an equal volume of solvent alone. The oviposition deterrent index (ODI) was calculated for each test material according to the formula: $ODI = [(C - T)/C + T] \times 100$, where C and T are the numbers of eggs laid on control and treated plants, respectively (Lundgren, 1975). The ODI was calculated for each replication, and a paired t test was used to analyze the results for each compound tested.

Extraction and Isolation of Active Material. Fresh foliage of *E. cheiranthoides* was added to boiling ethanol, and after ca. 5 min the mixture was cooled and homogenized in a Waring blender. After filtration through glass wool, the ethanolic extract was evaporated to dryness, and the residue was sequentially extracted with hexane and water. The aqueous deterrent material was then extracted into *n*-butanol (Renwick and Radke, 1987) and, after evaporation of the butanol, the total residue was taken up in water for HPLC. Separation into fractions for bioassays was accomplished with a C_{18} reversed-phase column (Varian MCH-10, 30 cm \times 8 mm) at a flow rate of 3 ml/min. A water-acetonitrile gradient was used throughout and was adjusted to provide better resolution of active fractions as the isolation progressed. Chromatograms were initially obtained with UV detection at 254 nm, and later a diode array detector was used to monitor the eluate at 219 nm, based on the absorption maximum of active compounds.

Thin-layer chromatography (TLC) of active fractions was carried out on 10 \times 20 cm, 250- μ m thick, Whatman K6 silica gel plates. The solvent system consisted of ethyl acetate-methanol-water (8 : 1 : 0.8), and spots were visualized with ceric sulfate for glycosides or with Kedde's reagent for cardenolides (Krebs et al., 1969). Purification of individual compounds in the active HPLC fractions required separation on an open column of Woelm silica gel, 32-63 mesh, eluted with a gradient of ethyl acetate saturated with water and 0-15% methanol.

UV spectra of isolated compounds were obtained from the diode array detector (Hewlett Packard model 1040A) in water-acetonitrile and with a Perkin Elmer model Lambda 5 spectrophotometer in methanol solution.

RESULTS

Bioassays of extracts of *E. cheiranthoides* as oviposition deterrents to *P. rapae* confirmed our previous observation that the activity can be removed from aqueous extracts by *n*-butanol (Renwick and Radke, 1987). When tested in this

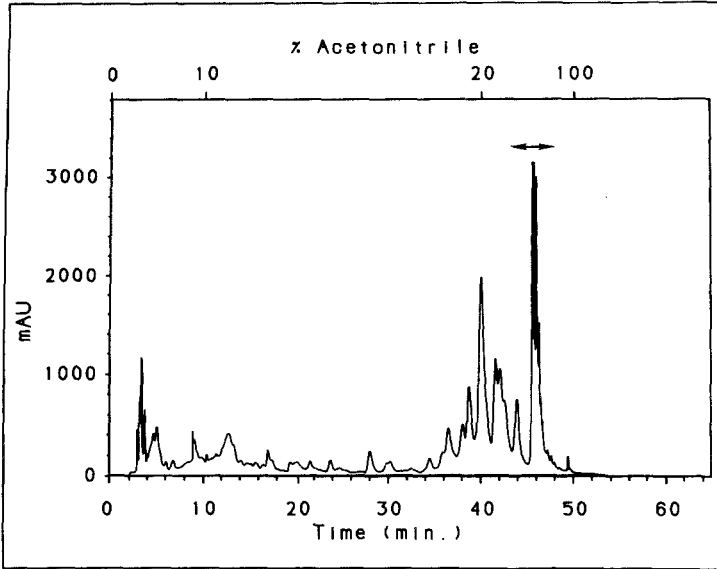


FIG. 1. Initial HPLC separation of butanol extract of *E. cheiranthoides*. Deterrent activity indicated by double arrow. UV monitoring at 254 nm.

study at 3 gram leaf equivalents (GLE) per plant, butanol extracts gave an ODI of 84.7. In 10 replications, a total of 108 eggs were laid on treated cabbage plants, whereas 1302 were laid on the controls ($P < 0.001$, paired t test).

Initial HPLC runs using a rapid solvent gradient resulted in isolation of the active material as a relatively nonpolar fraction eluting with 50–70% acetonitrile (Figure 1). Subsequent runs used a slow solvent gradient from 15 to 33% acetonitrile to further separate constituents of the active fraction (Figure 2).

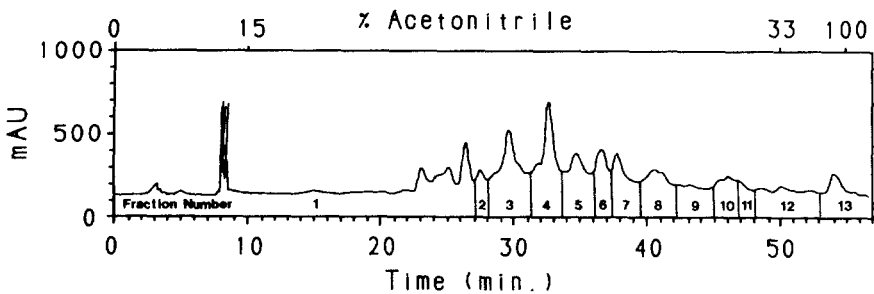


FIG. 2. HPLC separation of active fraction of *E. cheiranthoides* collected from initial HPLC run (as shown in Figure 1). UV monitoring at 254 nm.

TABLE 1. MEAN OVIPOSITION DETERRENT INDICES (ODI)^a OF *E. cheiranthoides* FRACTIONS COLLECTED FROM HPLC (FIGURE 2), IN BIOASSAYS WITH *P. rapae*

Fraction No.	ODI	Fraction No.	ODI
1	14.1	8	62.0
2	1.1	9	35.0
3	-20.2	10	53.0
4	27.0	11	44.0
5	78.0	12	25.0
6	96.0	13	26.0
7	73.0	—	—

^aOviposition deterrent index = $(C - T/C + T) 100$. *C* = eggs laid on control plant; *T* = eggs laid on treated plant. Mean values were obtained from two replications at 10 gram leaf equivalents/plant.

Several fractions from these separations were deterrent (Table 1), but most of the activity was concentrated in fractions 5–8. Better resolution in the region of high activity was obtained from the same material using a different solvent gradient that increased slowly from 15 to 28% acetonitrile (Figure 3). UV monitoring of the eluate at 254 nm showed little absorption for the active fractions, but when a wavelength of 219 nm was used, three prominent peaks were obtained (Figure 3).

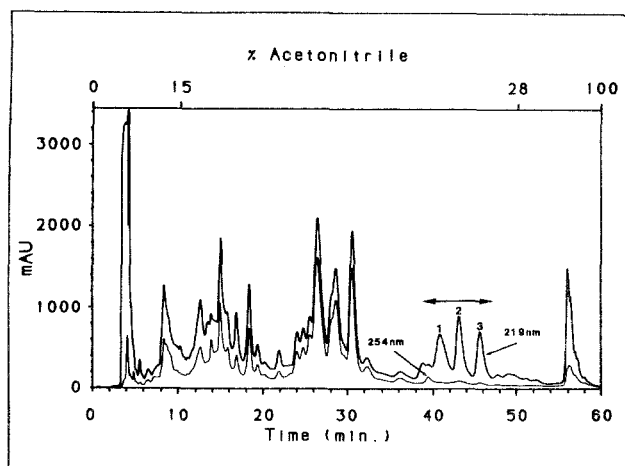


FIG. 3. Final HPLC separation of *E. cheiranthoides* deterrent fraction with UV monitoring at 254 and 219 nm. Active region indicated by double-headed arrow.

The UV spectra of the isolated prominent compounds (1, 2, and 3) were almost identical, with a λ_{max} at 216–219 nm, which is typical for a butenolide ring. TLC of the individual compounds collected from HPLC indicated the need for further purification (Figure 4), but visualization of spots with $\text{Ce}(\text{SO}_4)_2/\text{H}_2\text{SO}_4$ indicated that all the compounds were glycosides. Visualization of the spots with Kedde's reagent gave an intense purple color, which is typical for cardenolides.

Purification of the individual compounds on an open silica gel column and a final clean-up by HPLC resulted in the isolation of milligram quantities of pure compound 2 for bioassays and further characterization. The deterrent activity of compound 2 was compared with that of commercially available cardenolides at 2 concentrations (Table 2). Digitoxin, ouabain, and helveticoside were completely inactive, but compound 2 was deterrent at both 0.1 and 0.01 mg/plant. Cymarín was the only known cardenolide among those tested to show similar deterrent activity.

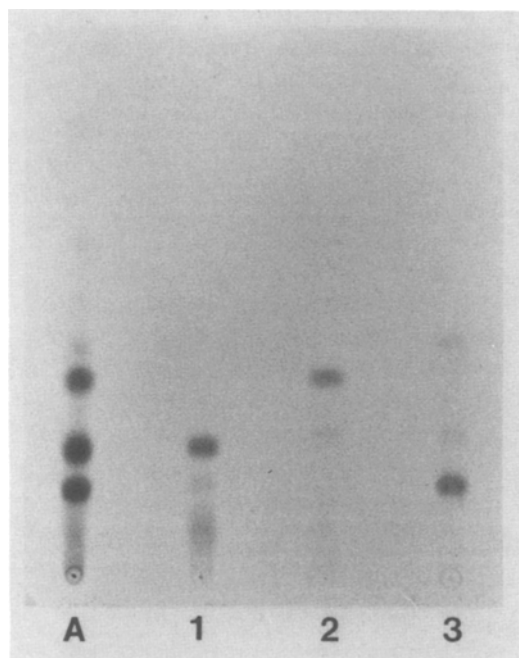


FIG. 4. TLC of active fraction (A) and individual peaks (1, 2 and 3) isolated in final HPLC separation.

TABLE 2. OVIPOSITION BY *Pieris rapae* ON CABBAGE TREATED WITH KNOWN CARDENOLIDES OR COMPOUND 2 FROM *E. cheiranthoides*

Test material	Total number of eggs laid ^a		Mean ODI	<i>P</i> ≤ (paired <i>t</i> test)
	Treated	Control		
Digitoxin				
0.1 mg/plant	408	498	9.9	0.375
0.01	518	595	7.0	0.375
Ouabain				
0.1	497	514	1.7	0.375
0.01	566	538	-2.5	0.375
Helveticoside				
0.1	541	662	10.0	0.1
0.01	906	1174	12.8	0.375
Cymarín				
0.1	192	650	54.4	0.025
0.01	395	679	26.4	0.005
Comp. 2				
0.1	210	725	55.1	0.0005
0.01	131	363	47.0	0.05

^aTotals from six replications.

DISCUSSION

The results show that avoidance of *E. cheiranthoides* by ovipositing cabbage butterflies can be explained by the presence of specific cardenolides in the foliage. Cardenolides have been reported in *Erysimum* species (Singh and Rastogi, 1970; Rodman et al., 1982), but their possible role in the defense of these plants against insects was not demonstrated until recently. Rothschild et al. (1988) isolated and characterized a cardenolide from Siberian wallflower, *Cheiranthus allionii*, that deters oviposition by *Pieris brassicae* in Europe. The host ranges of *P. rapae* and *P. brassicae* are very similar, and our results along with those of Rothschild et al. (1988) suggest that the two cabbage butterfly species rely on similar cues to reject unsuitable crucifers.

The idea that atypical secondary compounds in crucifers serve to protect certain species from herbivory provides a popular explanation for the escape of those plants from insect feeding (Feeny, 1977). Many attempts have been made to test this idea, usually by adding known compounds to insect diet (Nielsen,

1978; Usher and Feeny, 1983). Other studies have aimed to show the positive effects of typical compounds such as glucosinolates in the process of host finding and acceptance, often using relatively high concentrations of known compounds (reviewed by Schoonhoven, 1972). Our approach in studying the oviposition deterrents from *E. cheiranthoides* has been to isolate systematically the active material by solvent extractions and chromatographic separation, using bioassays to monitor the progress.

Our bioassays of known cardenolides as oviposition deterrents indicate that activity is likely to be restricted to a few specific members of the group. The four compounds tested along with compound 2 represent different groups of cardenolides based on the aglycones as well as type and number of sugars. Cymarín was active but is not known to be present in *E. cheiranthoides*. However, the results should pave the way for studies on structure–activity relationships. The final identification of the three major cardenolides in *E. cheiranthoides* is almost complete and will be reported separately.

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CHEMICAL CONSTITUENTS AND INHIBITORY ACTIVITIES OF ESSENTIAL OILS FROM *Cyperus brevifolius* and *C. kyllingia*

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Abstract—*Cyperus brevifolius* and *C. kyllingia* are two common weeds in Hawaii. The underground parts, including the rhizomes and roots, contain allelopathic essential oils, which may contribute to the aggressive spread of these weeds in grasslands and lawns. Although morphologically similar, *C. kyllingia* contains more essential oils than *C. brevifolius*. Also, the former is rich in terpenes, including α -cyperone, β -selinene, and α -humulene, while the latter is rich in C₁₇ to C₂₅ *n*-paraffins. These chemical differences agree with their inhibitory activities against indicator species and the general observation that *C. kyllingia* is the more prevalent weed of the two.

Key Words—*Cyperus brevifolius*, *Cyperus kyllingia*, weeds, allelopathy, inhibition, essential oils, terpenes, *n*-paraffins.

INTRODUCTION

Cyperus brevifolius (Ruttb.) Hassk (synonym, *Kyllingia brevifolius* Rottb.) is a perennial weed widely distributed in tropical and subtropical regions. It is found in open waste areas, grasslands, and lawns, and has long been established in Hawaii. Another weed species, *C. kyllingia* f. *Humulis*, which presents persistent problems in lawns and gardens, has been considered as a dwarf form of *C. brevifolius* (Neal, 1965). According to our observations, however, *C. kyllingia* is more commonly distributed than *C. brevifolius* on Oahu, Hawaii, and the height of *C. kyllingia* is variable, depending on various environmental fac-

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tors. While similar in many morphological characteristics, a distinctive difference between the two species is that *C. brevifolius* has green globular flower heads, and *C. kyllingia* has white ones. Common names for these two weeds, therefore, are "green head" and "white head" kyllingia, correspondingly.

Both *C. brevifolius* and *C. kyllingia* have been observed to make spot invasions upon grasslands and lawns followed by circular spread, and establish patches or colonies (Sakai and Kawanabe, 1981; Barnes and Chandapillai, 1972). These aggressive and persistent characteristics are similar to those of purple nutsedge (*C. rotundus*), whose underground rhizomes and tubers contain several phytotoxic sesquiterpenes. Composition of the terpene oils in tubers has been examined, and four major chemotypes has been reported so far (Komai, et al., 1977; Komai and Ueki, 1981; Komai and Tang, 1988). Neither *C. brevifolius* nor *C. kyllingia* forms any tubers. However, their rhizomes and roots have a strong terpenelike aroma, and their underground parts have been used as perfume and herbal medicine in some countries (Neal, 1965).

Many terpenoids are phytotoxic and have been suggested to be allelopathic or inhibitory to the growth of nearby plants or microorganisms (Putnam and Tang, 1987). In this report, the essential oils from *C. kyllingia* and *C. brevifolius* were obtained from the fresh rhizomes and roots. The chemical characteristics and plant growth inhibitory activities of the two morphologically similar weeds were compared.

METHODS AND MATERIALS

Isolation and Identification of Essential Oils. Two kilograms each of roots and rhizomes of mature, flowering *Cyperus brevifolius* and *C. kyllingia* were collected on the Manoa campus, University of Hawaii. After removal of soil and debris, the fresh tissues were crushed and extracted with 8 liters of *n*-hexane. The extract was dried over anhydrous sodium sulfate and the bulk of the solvent evaporated with a rotary evaporator under reduced pressure. Further concentration was carried out under a stream of N₂. Both concentrates from the two species had a strong, typical terpene aroma and were used for instrumental analyses.

GC was performed on a Spectra-Physics SP-7100 gas chromatograph equipped with a flame ionization detector and a 20-m × 0.25-mm-ID, DB-5 fused silica capillary column (J&W Scientific). The oven temperature was programmed from 90 to 250°C at 4°C/min. For GC-MS analysis a model HP-5730 gas chromatograph-mass spectrometer was used. Conditions for GC of the GC-MS were similar to those of the GC analysis. For the MS, 70 eV and 300 mA electron energy were used. The ion-source temperature was 200°C.

Compounds were identified by the comparison of GC retention times and mass spectra with those of the authentic compounds.

Inhibitory Activities of Essential Oils. Inhibition of lettuce (*Lactuca sativa* L. Anuenue) seed germination was performed in a Petri dish containing a Whatman No. 1, 5-cm-diameter filter paper. Two mls of the *n*-hexane solution with a known concentration of the essential oils was added to the paper and the hexane evaporated in a hood. The paper was moistened with 2 ml of distilled water, and 20 lettuce seeds were placed in the covered Petri dish in the dark at 23°C. The rate of germination was determined after 48 hr.

Inhibition of the lettuce seedling growth (Table 2) was performed by transplanting 10 pregerminated seeds to a Petri dish containing a filter paper and 2 ml of the aqueous suspension of a known quantity of essential oil. Hypocotyl and radicle length were measured four days after incubation, and the inhibitory activity was expressed as percent growth of those of the control (i.e., without essential oil).

Methods for determining inhibition of oat (*Avena sativa* L.) seedlings (Table 3) were similar to those of the lettuce except that a 9-cm Petri dish with filter paper and 4 ml of aqueous suspension of the essential oils were used instead. The lengths of leaf sheath and radicle were measured after four days. Inhibitory effects were also tested using fumigation (Table 4). A 3-cm-diameter center well containing 5 ml of an aqueous suspension with known amounts of the essential oils was used as the source of the volatile inhibitors. Distilled water was added to the filter paper disk for the germination of seeds. All experiments were performed in the dark at 23°C in covered Petri dishes.

RESULTS AND DISCUSSION

Composition of Essential Oils and Chemotaxonomy. Yield of the essential oils from underground parts of *C. kyllingia* was substantially greater than that from the *C. brevifolius* (0.72% vs. 0.15%, on fresh weight basis). Table 1 shows the qualitative and quantitative data of the *n*-hexane extracts using capillary GC and GC-MS.

The essential oils of both species had a typical terpene aroma. *C. kyllingia* contained mainly sesquiterpenes; the two major ones were β -selinene (28.1%) and α -cyperone (30.9%). *n*-Paraffin content accounts for less than 7% and the most abundant one was C₁₉H₄₀ (6.7%). In contrast, the essential oil from *C. brevifolius* contained mainly C₁₇ to C₂₅ *n*-paraffins (ca. 58%). Only a trace of β -selinene was detected, and there was no α -cyperone. Thus, the two species can be easily distinguished by their gas chromatograms in case the flower heads are not present.

TABLE 1. COMPOSITION OF ESSENTIAL OILS IN RHIZOMES AND ROOTS OF *Cyperus brevifolius* AND *C. kyllingia*^a

Compound	<i>C. brevifolius</i>	<i>C. kyllingia</i>	Methods ^b
α -Copaene	t	8.5	1,2
Cyperene	2.8	2.2	1,2,3
β -Elemene	7.3	3.3	1,2
Caryophyllene	5.4	0.6	1,2
α -Humulene	7.8	11.3	1,2,3
β -Selinene	t	28.1	1,2,3
δ -Cadinene	9.5	4.4	1,2,3
Calamenene	1.3	0.6	1,2
C ₁₇ H ₃₆	7.8	n	1,2
α -Cyperone	n	30.9	1,2,3
Patchoulenone	1.4	2.0	1,2,3
C ₁₈ H ₃₈	6.2	n	1,2,3
<i>E</i> -nerolidol	3.8	n	1,2,3
C ₁₉ H ₄₀	15.6	6.7	1,2,3
C ₂₁ H ₄₄	16.2	t	1,2,3
C ₂₃ H ₄₈	7.9	t	1,2,3
C ₂₅ H ₅₂	4.6	n	1,2,3

^aPercent of the total volatile compounds as determined by GC analysis. t = trace; n = none detected.

^bMethods used for identification: 1 = retention time on GC; 2 = GC-MS; 3 = cochromatography with the authentic compound.

Earlier, a survey by Komai (1982) reported that the essential oils from six *Cyperus* species, i.e., *difformis* L., *haspan* L., *polystachyos* Rottb., *globosus* All., *sanginolentus* Vahl., and *brevifolius* Rottb. were rich in paraffins (C_nH_{2n+2}, N = 19–27). These were wetland or paddy species without tubers. Except for *C. brevifolius*, which is perennial, all the others are annual sedges.

Comparing *Cyperus* spp. of dryland habitat, such as *C. rotundus* and *C. kyllingia*, it is apparent that the paraffin content is higher in the above-mentioned wetland species. This striking difference is of interest from both biochemical and ecological standpoints. Biochemically, paraffins are synthesized from the fatty acid pathway while terpenes are from the mevalonic acid pathway. Therefore, the paraffin-rich and the terpene-rich *Cyperus* species differentiated biochemically at an early stage of acetate utilization, suggesting basic genetic differences in photosynthate utilization as well as adaptation strategy. These differences may serve as chemotaxonomical bases for the division of these two types of *Cyperus* spp.

Plant Growth Inhibitory Activities of Essential Oils. The essential oils were not inhibitory to the germination of either lettuce or oat seeds; even at the high concentration of 1000 ppm, 100% germination was observed. However, the

TABLE 2. INHIBITION OF LETTUCE (*Lactuca sativa* L.) SEEDLING GROWTH BY AQUEOUS SUSPENSION OF ESSENTIAL OILS OF *Cyperus brevifolius* AND *C. kyllingia*^a

Conc. (ppm)	<i>C. brevifolius</i>		<i>C. kyllingia</i>	
	R	H	R ^b	H
50	89.7	93.5	44.0*	52.7*
100	89.8	69.9*	50.9*	45.7*
250	73.2*	67.2*	47.4*	39.8*
500	66.2*	60.2*	(32.3)*	34.4*
1000	64.2*	61.3*	(29.4)*	27.4*

^aExpressed in Percent Length of radical (R) and hypocotyl (H) of the treated in comparison to those grown in distilled water control. Average of three treatments; measured after four days. For distilled water control, the hypocotyl length = 18.6 mm; radicle length = 23.4 mm. *Significantly different from the control at more than 95% confidence level.

^bData in parentheses indicate that radicles withered after browning.

lettuce seedling growth rate (Table 2) was reduced by more than 50% of that of the water control when treated with 50 ppm essential oil from *C. kyllingia*. *C. brevifolius* showed a much weaker effect, reducing only to less than 10% of the control. Even at the high concentration of 1000 ppm, the growth reduction was less than 40%, while severe inhibition causing browning and withering of the radicles was observed in seedlings grown in a suspension of *C. kyllingia* oil at 500 ppm. The higher activity of the essential oil from *C. kyllingia* is expected since it has a high ratio of terpenes. The essential oil from *C. brevifolius* has a high ratio of paraffins, which, unlike the terpenes, are nontoxic in general. Less severe effects were observed when lettuce seedlings were treated with the volatiles released from the aqueous suspension of the essential oils (Table 3), confirming the presence of volatile inhibitors under the treatment conditions.

TABLE 3. INHIBITION OF LETTUCE SEEDLING GROWTH BY FUMIGATION USING AQUEOUS SUSPENSION OF ESSENTIAL OILS OF *Cyperus brevifolius* AND *C. kyllingia*^a

Conc. (ppm)	<i>C. brevifolius</i>		<i>C. kyllingia</i>	
	R	H	R	H
100	115.3	84.1	106.4	56.8*
500	96.6	82.8	79.8	33.9*
1000	86.2	52.0* ^b	50.7*	22.0*

^aExpressed in percent length of radicle (R) and hypocotyl (H) of the treated in comparison to those grown without fumigation (control). Average of three treatments, measured after four days. For control, the hypocotyl length = 22.7 mm; radicle length = 20.3 mm.

^bSignificantly different from the control at more than 95% confidence level.

TABLE 4. INHIBITION OF OAT (*Avena sativa* L.) SEEDLING GROWTH BY AQUEOUS SUSPENSION OF ESSENTIAL OILS OF *Cyperus brevifolius* AND *C. kyllingia*^a

Conc. (ppm)	<i>C. brevifolius</i>		<i>C. kyllingia</i>	
	R	S	R	S
50	90.1	104.6	71.9*	109.4
100	81.8*	92.4	84.6	114.1
250	70.1*	72.3*	36.4*	75.1*
500	63.6*	65.4*	10.5*	31.4*
1000	71.6*	68.6*	8.9*	12.4*

^aExpressed in percent length of radicle (R) and leaf sheath (S) of the treated in comparison to those grown in water (control). Average of three treatments; measured after four days. For distilled water control, the leaf sheath length = 18.5 mm; radicle length = 32.4 mm.

^b*Significantly different from the control at more than 95% confidence level.

Similar differences were shown in the inhibition of oat seedling growth (Table 4). The oat radicle showed a higher susceptibility than the leaf sheath to the toxicity of the essential oil of *C. kyllingia*. For example, at 250 ppm, the growth of radicle was reduced by 65% but the growth of the leaf sheath by only 25%. In general, roots are more susceptible to the inhibition of these essential oils, showing reduction of root hairs and browning in addition to growth.

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CYANOGLYCOSIDE GYNOCARDIN FROM *Acraea horta*
(L.) (LEPIDOPTERA: ACRAEINAE)
Possible Implications for Evolution
of Acraeine Host Choice

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Abstract—All stages in the life cycle of *Acraea horta* (L.) (Lepidoptera: Acraeinae) were found to release hydrogen cyanide (HCN) from their crushed tissues, and the source of cyanogenesis was present in the hemolymph of adults and larvae. Comparison with standards on thin-layer chromatograms (TLC) revealed the presence in adults of gynocardin, a cyclopentenyl cyanoglycoside also produced by the larval food plant, *Kiggelaria africana* L. (Flacourtiaceae). Analysis of adults reared on plant species (Passifloraceae) containing gynocardin and/or other cyanoglycosides suggested selective uptake of gynocardin by the larvae. This is the first demonstration of a cyanoglycoside, other than the acyclic linamarin and lotaustralin, occurring in Lepidoptera and the first evidence for the storage by *Acraea* butterflies of a plant-produced allelochemical. Possible implications for the understanding of the evolution of acraeine host choice are discussed.

Key Words—*Acraea horta*, Lepidoptera, Acraeinae, cyclopentenyl cyanoglycoside, gynocardin, *Kiggelaria africana*, sequestration, evolution.

INTRODUCTION

Unpalatable herbivorous insects that store the same toxic chemicals as their hosts often obtain these from the plants they eat (Brower and Brower, 1964; Rothschild, 1972). It was therefore surprising when evidence emerged sug-

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gesting that all three groups of Lepidoptera known to contain cyanoglycosides are an exception in this regard: the Zygaenidae and the nymphalid groups, Heliconiini and Acraeinae, feed selectively on plants containing cyanoglycosides and yet synthesize these compounds themselves.

This was first reported by Jones *et al.* (1962), who found that *Zygaena filipendulae* (L.) and *Z. lonicerae* (von Schev.) (Zygaenidae) release hydrogen cyanide (HCN) from their crushed tissues even when reared on acyanogenic plants. Davis and Nahrstedt (1979) subsequently identified the chemical basis of cyanogenesis in *Z. filipendulae* to be the cyanoglycosides linamarin and lotaustralin. Thereafter, one or both of these compounds were detected in the adults of five species of *Acraea* (see Table 3) and three species of *Heliconius* (Nahrstedt and Davis, 1981) and subsequently in *A. violae* (Fabricius) and in six additional genera of the Heliconiini (Nahrstedt and Davis, 1983). The major food plants of the Heliconiini and Acraeinae, the Passifloraceae and related families (Smiley, 1985; Ackery, 1987), produce cyanoglycosides with a cyclopentenyl moiety, suggesting that the acyclic linamarin and lotaustralin are synthesized by these butterflies. Nahrstedt and Davis (1983) confirmed this for *Heliconius melpomene* (L.) by demonstrating that larvae and adults incorporate radioisotope-labeled amino acids into linamarin and lotaustralin.

The question thus arises, why do cyanogenic Lepidoptera feed selectively on cyanogenic plants if not to obtain defensive chemicals? The recent discovery that *Zygaena trifolii* (Esper) and probably *Z. filipendulae* are capable, in addition to their *de novo* synthesis, of sequestering linamarin and lotaustralin (Nahrstedt and Davis, 1986), indicates that the association of these insects with cyanogenic plants does indeed involve the uptake and storage of plant-produced allelochemicals. Since there had been no demonstration of sequestration by the Heliconiini and Acraeinae, it was suggested that these groups originally colonized the largely competitor-free cyanogenic plants because their already established ability to synthesize cyanoglycosides preadapted them to cope with the toxicity of these compounds (Rothschild, 1972; Davis and Nahrstedt, 1985; Ackery, 1988). However, this hypothesis fails to explain why several species of *Acraea* show the patterns of host utilization typical of insects that obtain defensive chemicals from the plants they eat. These patterns include: (1) families and subfamilies of insects being more or less confined to a single group of plants with taxonomic and/or chemical affinities, and (2) different groups of insects having a similar pattern of host preference across several families of plants (Rothschild, 1972).

Acraea horta (L.) is one such species showing the patterns of herbivory typical of insects that sequester defensive chemicals from their hosts. With reference to the first of these patterns listed above, the larvae are monophagous on *Kiggelaria africana* L. (Flacourtiaceae) in their natural habitat and are also known to feed on introduced garden plants in the taxonomically related Passi-

floraceae (Pennington, 1978). Several *Acraea* species feed exclusively on one or both of these families and additionally, where available in their distribution range, on a third related family, the Turneraceae (Ackery, 1988). The Flacourtiaceae, Passifloraceae, and Turneraceae have biochemical affinities in that it is only from this group of plants that cyanoglycosides with the unusual cyclopentenyl structure are known (Seigler, 1975). With reference to the second of the patterns listed above, the Heliconiini show a similar pattern of host specificity to the Acraeinae, in which the larvae feed only on the Passifloraceae and Turneraceae (Smiley, 1985).

The circumstantial evidence therefore suggests that *A. horta* may obtain defensive compounds from their larval food plants. This would have implications for our understanding of how acraeine butterflies came to be associated with cyanogenic plants, and the purpose of the present study was therefore to investigate the source of cyanogenic compounds in *A. horta*.

METHODS AND MATERIALS

Test for Cyanogenesis. Eggs, larvae, pupae, and adults were collected from *Kiggelaria africana* trees in Newlands Forest, Cape Town. Larvae, pupae, and adults were tested for cyanogenesis by crushing the individual organisms in 3 ml of distilled water in a glass vial (50 × 10 mm) in which a strip of picrate paper was suspended before stoppering (Steyn, 1934); eggs were tested in the same way in batches of approximately 50. For larvae, the digestive tract was first dissected out in order to remove any residual plant tissue. In addition, the hemolymph of adults and larvae and the dissected wings, head, thorax, and abdomen of adults were tested separately. Hemolymph was extracted from the wing veins of adults and the body spines of larvae using 2- μ l glass pipets and the full pipets were crushed in vials containing picrate paper. The presence of hydrogen cyanide (HCN) was indicated by a change in the picrate paper from yellow to purple-brown. The time taken for this change to begin and the final intensity of the coloration are roughly proportional to the quantity of HCN released (Jones *et al.*, 1962; Gibbs, 1974). In all cases the test was conducted at 25°C and allowed to run for 24 hr. Each test was replicated at least 10 times on both male and female insects.

Identification of Gynocardin in A. horta. Gynocardin was identified from *A. horta* adults using correlative chromatography on thin-layer plates (TLC) (silica gel 60F, 254) and Whatman No. 1 paper (descending). Reference standards were pure gynocardin isolated from the leaves of *Kiggelaria africana* (Raubenheimer and Elsworth, 1988) and linamarin supplied by Professor A. Nahrstedt and Dr. R. H. Davis. The sandwich technique (Tantisewie *et al.*, 1969) was used to monitor cyanoglycosides on paper chromatograms, incor-

porating a crude enzyme extract from leaves of *K. africana* as a hydrolytic agent (Spencer and Seigler, 1982). On TLC, glycosides were visualized by spraying with cerium (IV) sulfate in 1.5 M H₂SO₄ followed by heating for 1–2 min on a hot plate; the cyanogenic nature of the glycosides was confirmed using the sandwich technique modified for use with TLC. The cyanoglycosides were applied to chromatograms as methanolic solutions. Solvent systems used are shown in Table 1.

Extraction of Gynocardin. To isolate the cyanogenic substance from *A. horta*, 235 (16 g fresh weight) adults were collected, frozen, and later macerated in boiling 96% ethanol. Since the test for cyanogenesis (see above) revealed no differences between the sexes, this sample contained approximately equal numbers of males and females. The resulting slurry was filtered and the filtrate defatted using petroleum ether, then dichloromethane, and dried under vacuum to a syrup (1.2 g). This was adsorbed onto 1.5 g silica gel (Merck 35–70 mesh), placed on a silica gel column (Merck 70–230 mesh, 60 g), and eluted using acetone–water (5:1). Fractions (15 ml) were collected and monitored for cyanoglycosides using cerium (IV) sulfate reagent and the modified sandwich method on TLC (see above). The cyanogenic fractions were combined, dried to a syrup,

TABLE 1. MOBILITY OF CYANOGLYCOSIDE EXTRACTED FROM *Acraea horta* ADULTS COMPARED WITH LINAMARIN AND GYNOCARDIN STANDARDS

Chromatographic system	Mobility (R_f)		
	Linamarin standard	Gynocardin standard	Extracted from <i>A. horta</i>
TLC			
Ethyl acetate–acetone–methanol (4:4:1)	0.49	0.41	0.41
Chloroform–methanol (5:2)	0.52	0.34	0.34
Butanol–acetic acid–water (2:1:1)	0.63	0.58	0.58
Butanol–ethanol–water (4:1:5)	0.52	0.50	0.50
Dichloromethane–ethanol (2:1)	0.62	0.53	0.53
Paper (descending)			
Butanone–acetone–water (15:5:3)	not measured	0.71	0.71
Butanol–acetic acid–water (2:1:1)	not measured	0.29	0.29
Acetone–water (5:1)	not measured	0.88	0.88

and partially purified using preparative plate chromatography (silica gel 60, Merck; ethyl acetate-acetone-methanol 4:4:1). The cyanogenic front ($R_f = 0.38-0.62$) was located by spraying a 1-cm-wide strip of the chromatogram with crude enzyme extract from *K. africana* and overlaying this with medicinal gauze and picrate paper (Steyn, 1934). The chromatogram, picrate paper, and gauze were enclosed between glass sheets and incubated at 30°C until a red band appeared on the yellow picrate paper, indicating the cyanoglycoside front. The cyanoglycoside was eluted using methanol.

Test for Sequestration. To investigate the possible sequestration of cyanoglycosides, *A. horta* were reared from eggs to the adult stage on the leaves of plants containing gynocardin and/or other cyanoglycosides. These plants, *Passiflora caerulea* L., *Tacsonia mollissima* Kunth., and *Tacsonia manicata* Juss. (Passifloraceae), were selected as those most readily eaten by fifth-instar larvae in a previous experiment (Raubenheimer, 1987). The butterflies were then extracted in ethanol and the resulting extracts compared for cyanoglycosides using TLC with the extracts of the respective food plants on which each batch of butterflies had been reared and with a gynocardin standard.

Owing to low survival rates on these plant species, extractions were limited to three butterflies per batch. It was thus not practical to purify the butterfly extracts chromatographically, as had been done to establish the presence of gynocardin in the wild butterflies (see above). The crude extracts were, therefore, filtered, dried under vacuum, and made up to equal concentration (weight/volume) in methanol before application to TLC for comparison. Cyanoglycosides were monitored using the sandwich method (Tantisewie et al., 1969). Hydrolysis of the cyanoglycosides was achieved by spraying the chromatograms with a mixture of crude enzyme extracts from *K. africana*, *P. caerulea*, *T. mollissima*, and *T. manicata*. This ensured that a negative result was due to the absence in the organism under test of cyanoglycoside and not of an appropriate hydrolytic enzyme (Nahrstedt et al., 1981).

RESULTS

Test for Cyanogenesis. All stages in the life cycle gave a strong positive result for cyanogenesis, with a color change beginning within 5 min and resulting in dark purple-brown within 30 min. The dissected wings, head, thorax, and abdomen of adults all gave a positive reaction with picrate paper, as did the hemolymph of adults and larvae. For hemolymph, however, the reaction took up to an hour to begin, resulting in a dark orange after 24 hr. This was suspected to result from the small quantities tested. There were no apparent differences between the sexes.

Identification of Gynocardin. Table 1 shows the mobility on various chro-

matographic systems of the cyanogenic compound extracted from *A. horta* adults, together with that of gynocardin and linamarin standards. These results indicate that the butterflies contained gynocardin, the same compound produced by their larval food plant.

Test for Sequestration. The results of the experiments to test for sequestration are presented in Table 2. *Tacsonia manicata* contained only gynocardin, as did the butterflies reared from the egg stage on this species. While the leaves of *Passiflora caerulea* contained gynocardin as a minor component (as gauged by intensity of development on TLC) as well as a second, less polar cyanoglycoside as a major component, the insects reared on this species contained only gynocardin. No cyanogenic activity resulted when the enzyme extract from the leaves of this species was added to the frass of larvae, suggesting that the major glycoside was not excreted. Neither this nor the cyanoglycoside extracted from *T. mollissima* (see below) were hydrolyzed by the enzyme extract from *K. africana*. Gynocardin was not detected from extracts of the leaves of *Tacsonia mollissima*, but a less polar cyanoglycoside, which ran concurrently with the major glycoside in *P. caerulea* (using TLC in butanol-acetic acid-water, 2:1:1), was present. No cyanogenic substance was detected on TLC plates from the extracts of *A. horta* adults reared from the egg stage on this species. When four intact adults reared from the egg stage on *T. mollissima* were crushed in separate vials containing picrate paper, two gave a slight positive reaction, with the color change beginning after 1 hr and resulting in a light orange after 24 hr, and two gave no reaction. This demonstrates that there was some potential for cyanogenesis even in insects reared on plants that, in other cases, resulted in acyanogenic butterflies. Controls reared on *K. africana*, *T. manicata*, and *P. caerulea* caused a color change within 5 min, resulting in dark purple-brown after 24 hr. Adults of larvae transferred from *K. africana* to *T. mollissima* in their second instar gave a final color change after 24 hr to a dark orange, indi-

TABLE 2. COMPARISON OF CYANOGLYCOSIDES EXTRACTED FROM *Acraea horta* ADULTS WITH THOSE EXTRACTED FROM LEAVES OF THREE PLANT SPECIES ON WHICH INSECTS HAD BEEN REARED FROM EGG STAGE^a

Plant species	Plant extract	R_f	Butterfly extract
<i>Tacsonia manicata</i>	0.58 ^b		0.58 ^b
<i>Passiflora caerulea</i>	0.58 ^b		0.58 ^b
	0.43		nd
<i>Tacsonia mollissima</i>	0.43		nd

^a Figures refer to R_f values (TLC, butanol-acetic acid-water 2:1:1); nd = not detected.

^b Identified as gynocardin using the chromatographic systems listed in Table 1.

cating levels of HCN intermediate to those discussed above. Addition of the enzyme extract from *T. mollissima* caused no further color change, thus confirming that the results reflected the levels of cyanoglycoside rather than the absence of appropriate hydrolytic enzymes. The low survival rates of insects reared on these plant species precluded any further quantification of these results.

DISCUSSION

Selective Sequestration of Gynocardin. Results of this study demonstrate that all stages in the life cycle of *Acraea horta* are cyanogenic. The chemical basis of this in the adults is gynocardin, which is biogenetically unrelated to linamarin and lotaustralin (Conn, 1979), the cyanoglycosides previously detected in several *Acraea* species (Table 3). It cannot, however, be ruled out that trace amounts of linamarin and lotaustralin were also present, and more sensitive techniques are needed to investigate this.

Gynocardin is also the cyanoglycoside produced by the larval food plant *Kiggelaria africana* (Raubenheimer and Elsworth, 1988), and the present results demonstrate that cyanogenesis in *A. horta* is dependent on the diet of these

TABLE 3. *Acraea* SPECIES INVESTIGATED FOR IDENTITY OF CYANOGLYCOSIDES CONTAINED, CYANOGLYCOSIDES DETECTED, AND GROUPS OF PLANTS ON WHICH LARVAE ARE KNOWN TO FEED^a

<i>Acraea</i> species	Cyanoglycosides detected	Known larval food plants ^d
		<u>Dicotyledons</u>
<i>A. horta</i>	gynocardin	Violales
<i>A. andromache</i>	neither linamarin nor lotaustralin detected ^b	Violales
<i>A. caldarena</i>	linamarin ^c	Violales
<i>A. natalica</i>	linamarin ^c	Violales
<i>A. onacaea</i>	linamarin ^c	Violales + Vitidaceae
<i>A. violae</i>	linamarin ^b	Violales + Malvales
<i>A. eponina</i>	linamarin and lotaustralin ^c	Malvales + Solanaceae
		<u>Monocotyledons</u>
<i>A. encedon</i>	linamarin and lotaustralin ^c	Commelinaceae

^aThe Malvales-feeding group and those feeding on monocotyledons are considered more recently evolved than the Violales-feeding group—see text for further discussion.

^bNahrstedt and Davis (1983).

^cNahrstedt and Davis (1981).

^dAckery (1988).

insects. Thus, adults reared on *Tacsonia mollissima*, which did not contain gynocardin, showed, at the most, low cyanogenic activity. It is unlikely that this was a result of some general dietary inadequacy due to *T. mollissima* being alien to the evolutionary history of *A. horta*, since adults reared on *Passiflora caerulea* and *T. manicata*, neither of which occur naturally in the distribution range of *A. horta*, were strongly cyanogenic; these plants however contained gynocardin. Similarly, those that fed as larvae on *K. africana* prior to being transferred to *T. mollissima*—and had thus been exposed to a dietary source of gynocardin—showed higher cyanogenic activity than those transferred as eggs. Together, this evidence suggests that *A. horta* obtains gynocardin from the larval food plants.

The glycoside responsible for cyanogenesis in *T. mollissima* did not appear in adults reared on this species, suggesting that the uptake of cyanoglycosides by *A. horta* is selective. Similarly, of the two cyanoglycosides produced by *P. caerulea*, adults reared on this species contained only gynocardin. Interestingly, crude enzyme extract of *K. africana* did not hydrolyze the second of these glycosides or the sole cyanoglycoside in *T. mollissima*. Therefore, assuming that the hydrolysis of gynocardin in *A. horta* is catalyzed by a similar enzyme system to that in *K. Africana* absorption of these compounds would not contribute to the cyanogenic capability of *A. horta*. Neither of these cyanoglycosides appeared in the frass of larvae fed the leaves of *P. caerulea* or *T. mollissima*, and it must therefore be concluded that they were metabolized by the insects.

There are interesting similarities in these results and those of von Euw et al. (1967). The tissues of *Poekilocerus bufonius* (Klug), an aposematic grasshopper, were found to contain two of the six cardenolides produced by their asclepiad food plant, suggesting that the remaining four were either excreted, metabolized, or converted in vivo by these insects. Hoppers reared on nonpoisonous plants contained approximately 10 times less cardenolide than those reared on toxic plants, and these levels were further reduced by a factor of about seven in their offspring. That these offspring contained the toxins at all suggests that cardenolides are carried over from the adult to the egg stages, and from eggs to the larvae. However, von Euw et al. (1967) contend that cardenolide levels in both generations were unexpectedly high for storage in the egg alone and postulate that additional factors such as de novo synthesis or bioaccumulation through cannibalism must contribute to these. Similarly, the residual cyanogenic activity in *A. horta* adults reared on *T. mollissima* in the present study may have been due to any one or a combination of these factors. Answers to such questions must, however, await detailed quantitative analyses.

Evolution of Host Choice. The demonstration of gynocardin in *A. horta* raises some interesting questions regarding the colonization of cyanogenic plants by acraeine butterflies. Rothschild (1971) first predicted, on the basis of their patterns of host choice, that acraeine butterflies would sequester cyanoglyco-

sides from the larval food plants. The subsequent evidence that cyanogenesis in these insects is based on de novo synthesis (see Introduction), led to the suggestion that ancestral Acraeinae initially synthesized cyanoglycosides and were thus preadapted to cope with the toxic effects of these compounds in plants (Rothschild, 1972; Davis and Nahrstedt, 1985; Ackery, 1988). With the discovery of gynocardin in *A. horta*, Rothschild's (1971) hypothesis may now be vindicated. Perhaps the original diversification of *Acraea* butterflies on cyanogenic hosts was based on sequestration of cyanoglycosides and the capability to synthesize linamarin and lotaustralin evolved subsequently.

Table 3 lists those species of *Acraea* that have been investigated to determine the identity of the cyanoglycosides they contain, together with the known feeding preferences of the larvae. While it is clear from this that the *Acraea* species for which we know the chemical basis of cyanogenesis are too few to allow any firm conclusions, the data presented nonetheless suggest some interesting patterns. *Acraea horta* is associated exclusively with the Violales, considered the ancestral larval food plants of the Acraeinae (Ackery, 1987). The larvae are only known to feed on the Flacourtiaceae and Passifloraceae (Pennington, 1978) and, by inference from several other *Acraea* species sharing a similar pattern of host choice (e.g., see Raubenheimer, 1987), it seems likely that the closely related Turneraceae would also be accepted as hosts if available. These families have strong biochemical affinities in their production of cyanoglycosides with a cyclopentenyl moiety. Furthermore, this biochemical similarity is probably an important factor in larval host choice, since *A. horta* larvae feed selectively on cyanogenic species from among a range of plants in the Flacourtiaceae and Passifloraceae (Raubenheimer, 1987). It may be significant that Nahrstedt and Davis (1983) failed to detect linamarin or lotaustralin in *A. andromache*, a species that is also only known to feed on the Violales (Table 3), despite the fact that the insects were cyanogenic; it would be interesting to analyze these insects for the presence of cyclopentenyl cyanoglycosides. There therefore exists in *A. horta* an interesting link among acraeine butterflies between primitive patterns of host choice, cyclopentenyl cyanoglycosides, and the storage of plant-produced allelochemicals.

Acraea encedon L., by contrast, is believed to synthesize cyanoglycosides since the adults contain linamarin and lotaustralin, which do not occur in their larval food plants (Nahrstedt and Davis, 1981). This species is also, as far as is known, unique among acraeine butterflies and, indeed, unusual among Lepidoptera, in that the larvae feed on a monocotyledonous family, the Commelinaceae (Table 3). Since, in the words of Ehrlich and Raven (1965), "All utilization of foods other than dicotyledons by butterfly larvae . . . is assumed to be the result of changes from an earlier pattern of feeding on dicotyledons," there exists good reason to consider as derived this singular choice of host plant. Similarly, *A. eponina* contains linamarin and lotaustralin and is not known to

feed on the Violales, but is strongly associated with the Malvales [only known to feed on a single family outside this order (Table 3)]. Of the three groups into which most *Acraea* species fall on the basis of host-plant selection—the Violales group, the Utricales group and the Malvales group,—the Malvales group is considered the most recently evolved (Ackery, 1987).

There is additional evidence linking de novo synthesis of cyanoglycosides by *A. encedon* to derived features. In 1969 Owen and Chanter (1969) reported that no cyanogenic activity was detected from the tissues of *A. encedon* adults but suggested that an analysis of the yellow foam produced from a gland in the thorax may yield different results. Owen (1970) subsequently reported that HCN is indeed released from this exudate. Such localization of toxic substances in insects is considered to be specialized (Brower and Brower, 1964) relative to the state in *A. horta*, which contain cyanoglycoside throughout their bodies. It might also be significant in this context that *A. encedon* was found by Nahrstedt and Davis (1981) to contain an appreciably higher concentration of cyanide than the other *Acraea* species analyzed. Interestingly, *A. eponina*, the only other species analyzed that does not feed on Violales, contained the second highest concentration.

Acraea horta and *A. encedon* represent two extremes in the possible “sequestration-as-ancestral” evolutionary sequence considered for the Acraeinae: those that feed exclusively on the Passifloraceae and related families (Violales) and contain cyanoglycosides of the structural group characteristic of these families, and those that feed on monocotyledons and synthesize linamarin and lotaustralin. *Acraea caldarena* Hewiston and *A. natalica* Boisduval—both of which contain linamarin and yet are only known to feed on Passifloraceae and related families (Table 3)—may represent an intermediate stage in this sequence. These butterflies have possibly evolved the biochemical machinery for synthesising linamarin without having evolved the sensory or ecological changes necessary for a switch to unrelated host plants. Indeed, there is good reason to believe that among Lepidoptera such a host change may be genetically conservative. Wiklund (1975), for example, has shown that choice of host plant by adult and larval *Papilio machaon* is controlled by separate genes, and a successful host switch would therefore rely on the unlikely event of two simultaneous and independent mutations. Either of these mutations occurring singly would result in eggs being deposited on plants on which the larvae do not feed, and thus would be rapidly selected out of the population.

We might nevertheless expect to find species intermediate in pattern of host utilization between *A. caldarena* and *A. natalica* on the one hand and *A. encedon* on the other, i.e., species that produce linamarin and/or lotaustralin and feed on Violales as well as on unrelated families. Indeed, *A. violae* feeds on the Passifloraceae as well as members of the Malvales and *A. onacaea* Hopffer feeds on the Passifloraceae, Turneraceae, and the disparate family, Vitidaceae (Ackery, 1988). Both species contain linamarin (Table 3).

If the ability to synthesize cyanoglycosides is indeed a recently evolved trait, derived from ancestral species that sequestered these compounds from plants then the following questions need consideration: (1) what was the evolutionary impetus for such a switch from sequestration to de novo synthesis; and (2) why did this switch involve a change from sequestering cyanoglycosides with a cyclopentenyl moiety to synthesizing the acyclic glycosides linamarin and lotaustralin? Concerning the first question, Bernays and Chapman (1978) have shown that acridoid grasshoppers that sequester toxins from their food plants, have significantly longer developmental times than others, and this they consider to be a result of the considerable metabolic expenditure involved in the uptake of toxic chemicals. However, little is known about the cost of de novo synthesis of cyanoglycosides, and a full answer to this question must therefore await further research. Regarding the second question, it is interesting that linamarin and lotaustralin are also the cyanoglycosides synthesized by the Heliconiini (Nahrstedt and Davis, 1981, 1983) and by the taxonomically distant Zygaenidae (Davis and Nahrstedt, 1979, 1982). These are also the most widespread cyanoglycosides among plants (Conn, 1979). Such convergence may be the result of some factor that lowers the cost or increases the benefit of manufacturing these relative to other cyanoglycosides. For example, valine and isoleucine, the respective precursors of linamarin and lotaustralin (Seigler, 1975), may be more freely available in insects and plants than is cyclopentenyl glycine, the nonprotein amino acid precursor of the cyclopentenyl cyanoglycosides (Conn, 1981).

On the other hand, *A. horta* emerges from Piere's (1984) cladistic analysis as one of the most recently evolved of the recognized *Acraea* species, and it is therefore difficult to accept on the basis of this species alone that sequestration is the ancestral state. But then, according to Piere's cladogram, so are all the known cyanoglycoside-synthesizing species considered recently evolved.

The demonstration of gynocardin in *A. horta* has therefore reestablished the question of why acraeine butterflies came to be associated with cyanogenic plants. If this association is indeed a relic of ancestral species that sequestered cyanoglycosides from their passifloraceous hosts, this may explain the resemblance of acraeine patterns of host choice to those of insects that obtain defensive compounds from the plants they eat (Rothschild, 1972). However, this suggestion is currently based on insufficient evidence and must await further studies into the relationship between food choice and the chemical basis of cyanogenesis for a wide range of *Acraea* species.

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DUFOUR GLAND CONTENTS OF THREE SPECIES OF *Myrmecia* (HYMENOPTERA: FORMICIDAE), PRIMITIVE ANTS OF AUSTRALIA

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Abstract—Chemical investigation of the Dufour gland contents of three species of *Myrmecia* by GC and GC-MS showed that *M. gulosa* and *M. nigriceps* contain predominantly linear hydrocarbons, whereas *M. pilosula* contains acetates, aldehydes, and propionates. The major components of *M. gulosa* and *M. nigriceps* are (Z)-8-heptadecene, pentadecane, and heptadecane. Their chemical profiles are similar to each other and to that of *Nothomyrmecia macrops*, the most primitive living ant, placed by itself in a separate subfamily. The major components of *M. pilosula* are hexadecyl acetate, (Z)-9-octadecenyl acetate, (Z)-11-eicosenyl acetate, and (Z)-11-eicosenal. Some geographical variation was observed in the relative proportions of the components. The chemotaxonomic significance for the primitive Australian ant subfamilies Myrmeciinae and Nothomyrmeciinae is considered. The possibility is discussed that 11-eicosenal or geranyl citronellol, minor components of the Dufour gland secretion of *M. gulosa*, may be responsible for the aggressive behavior released by the natural stinging secretions.

Key Words—Ant, Dufour gland, *Myrmecia*, Hymenoptera, Formicidae, hydrocarbons, acetates, eicosenal, chemotaxonomy.

INTRODUCTION

The Dufour gland is a saclike organ attached to the poison apparatus in all aculeate Hymenoptera. The function of its secretion remains unknown for many species, although in individual cases among ants it has been shown to contain a trail pheromone, a home-range or territorial marking pheromone, or to have

other pheromonal properties. Its contents have been examined in a number of ant species, and in every case they have been shown to be a mixture of hydrocarbons or oxygenated hydrocarbon derivatives (Attygalle and Morgan, 1984). These mixtures are species specific and can be used to separate morphologically similar species. The contents may therefore be a useful systematic and taxonomic indicator. Howse and Bradshaw (1980), in considering the phylogeny of Myrmeciinae and Ponerinae, pointed to the need for "chemical studies [of the exocrine secretions] of the more primitive subfamilies . . . to shed light on the interrelationships."

Ants of the subfamily Myrmeciinae are found only in Australia and New Caledonia, and the only living genus of the subfamily is *Myrmecia*. The genus contains some of the most primitive ants known (Clark, 1951; Greenslade, 1979). They are usually large (13–38 mm in length), very agile, aggressive when disturbed, and have a sting-delivered venom of a proteinaceous type (Cavill et al., 1964). Robertson (1971) studied the pheromones involved in the aggressive behavior of *Myrmecia gulosa*. She observed an alarm effect resulting from the natural stinging secretions and determined that the pheromone was produced in the Dufour gland. Cavill and Williams (1967) examined *M. gulosa* Dufour glands and identified six aliphatic hydrocarbons, including (*Z*)-8-heptadecene (62%), pentadecane (17%), and heptadecane (4%). Robertson (1971) concluded that the alarm pheromone was probably a minor component in the gland, not yet isolated. Other exocrine secretions of *M. gulosa* have been studied, including their behavioral effects (Cavill and Robertson, 1965; Robertson, 1971). Chemical investigations have shown that the head (mandibular gland) contains 2,5-dimethyl-3-*n*-propylpyrazine (Brophy and Nelson, 1985) and the cuticular wax contains C₂₆–C₃₄ monomethyl alkanes and C₁₉–C₂₉ normal paraffins (Cavill et al., 1970).

In the present work, the Dufour gland contents of *M. gulosa* are reexamined using GC-MS and those of *M. nigriceps* and *M. pilosula* are investigated for the first time.

METHODS AND MATERIALS

Insect Material. Workers of *Myrmecia gulosa* (Fabricius) from Sydney, *M. nigriceps* Mayr from Black Mountain, Australian Capital Territory, and *M. pilosula* (F. Smith) from Mongarlowe, New South Wales, and from Hobart, Tasmania, identified by Dr. R.W. Taylor, were collected during February 1987. They were taken to Canberra (CSIRO) and there anaesthetized and killed by placing for 3 min in a Biofreezer at -50°C . The Dufour glands were removed by dissection under water. They were immediately sealed, either individually or in groups, in soft-glass capillaries (2 cm \times 2 mm), and kept cool until brought by air to Keele.

Instrumentation. Gas chromatography was carried out on a Carlo Erba Fractovap 4160 series instrument with a flame ionization detector and a Shimadzu Chromatopac C-R3A data processor. A fused silica capillary column (25 m \times 0.32 mm) coated with OV-1 stationary phase of 0.4 μ m film thickness was used for the analysis. Helium was used as the carrier gas at a flow rate of 1.0 ml/min.

The capillary tubes containing the samples were kept in the solid injector (Morgan and Wadhams, 1972) in the injection port at 220°C for 2 min before crushing. The split vent was kept closed during the injection and opened after 1 min. The oven temperature was initially 100°C and increased at a rate of 6°C/min to 270°C. Seven single glands of *M. gulosa*, 13 of *M. nigriceps*, and 15 of *M. pilosula* (seven from New South Wales and eight from Tasmania) were analyzed in this way. The absolute quantity of each component was determined by using a solution of pentadecane in hexane as an external standard.

Two or three single glands and samples of three or five glands for each species were analyzed by GC-MS, on a Hewlett Packard 5890 gas chromatograph and 5970 mass selective detector with HP59970C ChemStation. A fused silica capillary column (12 m \times 0.2 mm) coated with HP-1 (cross-linked methylsilicone gum \cong OV-1) of 0.33 μ m film thickness was used. The carrier gas was helium at 10 psi column head pressure (\cong 1 ml/min flow rate). The samples were introduced by the solid injection method described above. The oven temperature was initially 60°C and increased at a rate of 4°C/min to 250°C. The mass selective detector was set to monitor m/z 35–350 in the scan mode (\cong 1.5 scans/sec) under "Autotune" conditions using 70-eV ionization.

Determination of Double-Bond Position. A hexane extract (15 μ l) of one Dufour gland of *M. nigriceps* or five glands of *M. pilosula* were placed with dimethyl disulfide (10 μ l, Aldrich) and ethereal iodine (2 μ l, 0.25 M) in the bottom chamber of a Wheaton-Keele microreactor vial (Wheaton Scientific, Millville, New Jersey). This was closed with a Teflon-lined cap and kept overnight at 40°C (Attygalle and Morgan, 1986; Buser et al., 1983). Analysis of the reaction mixture was performed by GC-MS as above with a syringe injection. The temperature program was initially 80°C and increased at a rate of 8°C/min to 270°C, and the mass selective detector was set to monitor m/z 35–450.

The same reaction was carried out on a hexane solution of oleyl acetate (Sigma) and on a mixture of (*Z*)- and (*E*)-9-octadecenyl acetate formed by shaking the oleyl acetate (20 mg) in hexane (1 ml) solution with 5 μ l of Poutet's reagent [mercury (0.5 ml) dissolved in concentrated nitric acid (8 ml)] (Griffiths and Hilditch, 1932).

To determine the double-bond position of eicosenal, the aldehyde group was first reduced to the alcohol. The secretion from nine Dufour glands of *M. pilosula* workers was extracted with hexane (25 μ l), and the hexane was evap-

orated by a gentle stream of nitrogen and replaced by ethanol (15 μ l). Sodium borohydride (5 mg) followed by water (3 μ l) was added and the mixture agitated. The product was extracted into hexane (15 μ l) in a Keele Microreactor (Wheaton Scientific) and the reaction with dimethyl disulfide performed as above.

RESULTS

M. gulosa. Analysis of a sample of three Dufour glands of *Myrmecia gulosa* by GC-MS revealed 27 identifiable components (Figure 1). The mixture comprises mainly unsaturated and saturated linear hydrocarbons in the range C_{13} -

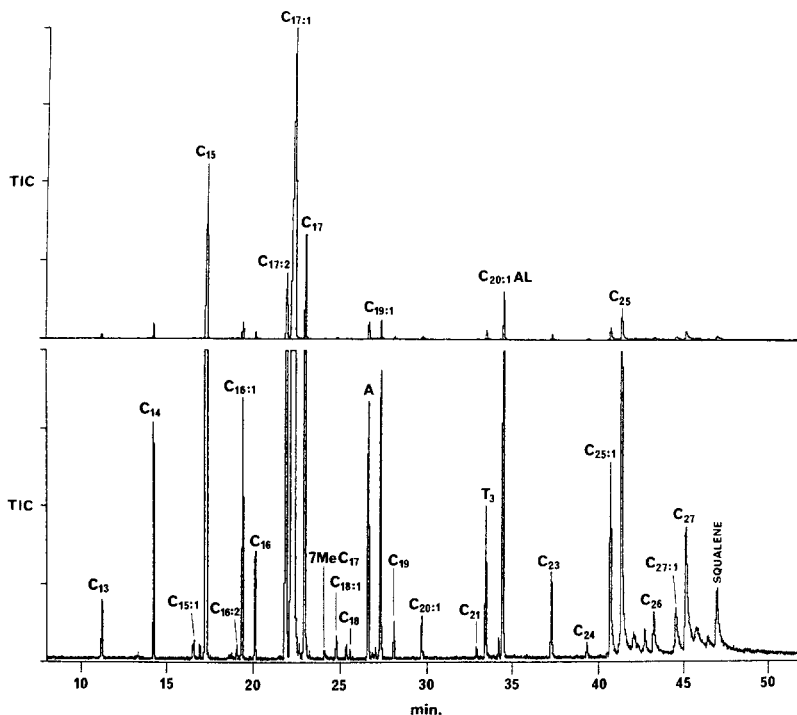


FIG. 1. Total ion chromatograms (TIC) from the GC-MS analysis of three Dufour glands of workers of *Myrmecia gulosa*, showing the minor as well as the major components. The symbols give identification, e.g., C_{15} is pentadecane, $C_{17:1}$ is heptadecene, $C_{16:2}$ is hexadecadiene, $C_{20:1AL}$ is eicosenal, T_3 is geranylcitronellol. The glands were chromatographed, using a solid injection technique, on a 12-m \times 0.12-mm fused silica capillary column coated with HP-1. The oven temperature was increased from 60°C at 4°C/min to 250°C, then held at 250°C.

C₂₇ (odd numbers predominating). Four of the minor components are not linear hydrocarbons. These are 7-methylheptadecane, eicosenal [M⁺ 294(0.2), 276(0.4), 135(3), 121(5), 111(9), 109(7), 98(17), 97(18), 96(14), 95(17), 83(30), 81(28), 69(49), 67(39), 57(41), 55(100), 43(59), 41(89)], an unidentified unsaturated compound A [tr 26.6 min, KI 1840, M⁺ absent, 217(0.6), 189(1), 175(1), 162(2), 150(4), 133(4), 122(7), 119(9), 105(15), 95(11), 93(29), 91(34), 81(25), 80(63), 79(95), 77(25), 67(72), 55(52), 43(32), 41(100), 29(41)] and a diterpene T₃. The mass spectrum of T₃ shows good agreement with that of all-*trans*-geranylcitronellol found in *Bombus lapponicus* (Hymenoptera: Apidae), which itself was identical with a synthetic sample (Bergström and Svensson, 1973). Squalene is probably not part of the Dufour gland secretion and may arise from the tissue (see Billen et al., 1988).

The quantification of the major components by GC is summarized in Table 1. We frequently find the size of Dufour glands varies considerably between individuals in a species. Here the variation was particularly marked, with the

TABLE 1. QUANTIFICATION OF MAJOR COMPONENTS OF DUFOUR GLANDS OF WORKERS OF *Myrmecia gulosa*

Compound	Mean composition by weight (ng/ant ± SD)	Mean percentage by weight (% ± SD)
Tridecane	100 ± 100	0.6 ± 0.6
Tetradecane	180 ± 140	0.6 ± 0.2
Pentadecane	4,300 ± 2,800	16.5 ± 3.2
Hexadecadiene	24 ± 25	0.2 ± 0.1
Hexadecene	520 ± 550	1.9 ± 1.2
Hexadecane	160 ± 140	0.6 ± 0.2
Heptadecadiene	1,750 ± 1,100	7.0 ± 0.4
(Z)-8-Heptadecene ^a	12,600 ± 8,100	50.4 ± 4.7
Heptadecane	1,600 ± 920	6.6 ± 1.6
Octadecene	320 ± 380	0.9 ± 0.8
Octadecane	23 ± 10	0.2 ± 0.1
Unknown 'A'	540 ± 380	2.3 ± 0.5
Nonadecene	560 ± 640	2.0 ± 1.2
Nonadecane	70 ± 60	0.4 ± 0.2
Eicosene	80 ± 60	0.6 ± 0.5
Geranylcitronellol (T ₃)	2,400 ± 3,400	5.9 ± 5.9
Eicosenal	650 ± 700	3.1 ± 1.7
Tricosane	35 ± 10	0.5 ± 0.1
Pentacosene	110 ± 40	1.4 ± 0.9
Pentacosane	520 ± 660	2.3 ± 0.9
Total	25,700 ± 18,000	

^aPosition and geometry determined by Cavill and Williams (1967).

total amount of material in individual workers' glands varying from 6 to 55 μg . However, when the percentage composition is calculated for each individual, and the mean and standard deviation calculated for each component, it is seen that the proportions of the major components do not vary very much. The major components are heptadecene (50.5%), pentadecane (16.5%), heptadecadiene (7.0%), and heptadecane (6.6%).

M. nigriceps. A GC-MS total ion chromatogram of the Dufour gland contents of *M. nigriceps* is shown in Figure 2 and the quantification of the major components summarized in Table 2. Twenty-eight components have been identified, and the composition is very similar to that of *M. gulosa*. However, in addition to eicosenal, five other linear aldehydes (pentadecanal, hexadecanal, heptadecanal, octadecanal, and octadecanal) and two more terpenes, T₁ [tr 24.8 min, KI 1770, M⁺ absent, 219 (0.4), 191 (0.6), 149 (0.6), 136 (4), 123 (5),

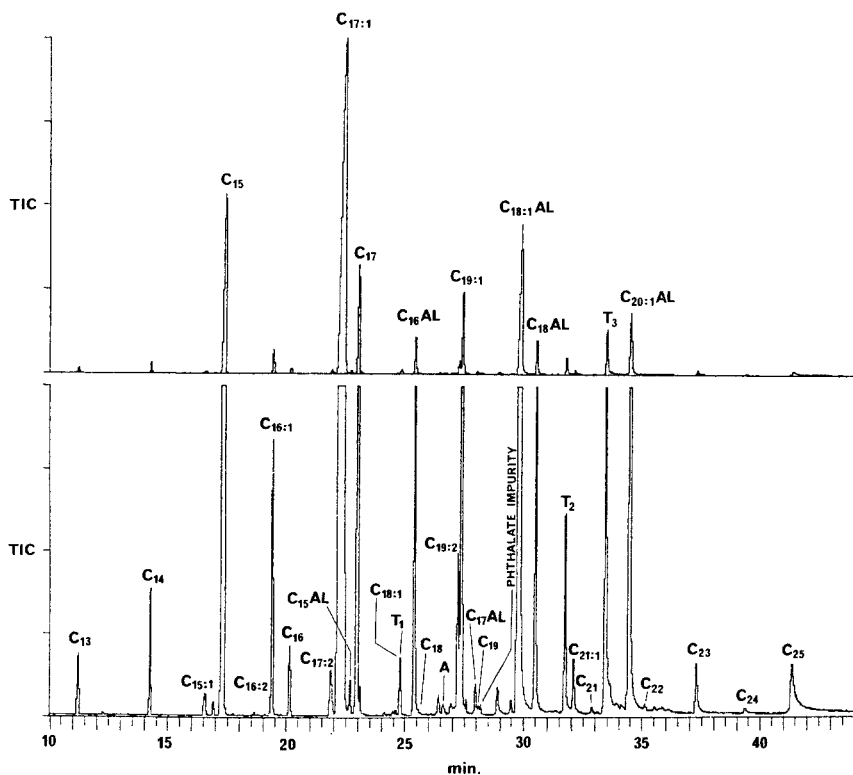


FIG. 2. Total ion chromatograms from the GC-MS analysis of the Dufour gland of a worker of *Myrmecia nigriceps*. Symbols and conditions as in Figure 1. T₁ and T₂ are unidentified terpenes.

TABLE 2. QUANTIFICATION OF MAJOR COMPONENTS OF DUFOUR GLANDS OF WORKERS OF *Myrmecia nigriceps*

Compound	Mean composition by weight (ng/ant \pm SD)	Mean percentage by weight (% \pm SD)
Tridecane	50 \pm 30	0.1 \pm 0.04
Tetradecane	150 \pm 110	0.5 \pm 0.2
(Z)-7-Pentadecene	70 \pm 60	0.2 \pm 0.2
Pentadecane	4,700 \pm 2,700	15.4 \pm 4.4
Hexadecadiene	26 \pm 23	0.1 \pm 0.03
(Z)-7-Hexadecene	430 \pm 320	1.4 \pm 0.4
Hexadecane	130 \pm 100	0.4 \pm 0.1
Heptadecadiene	1,200 \pm 800	3.0 \pm 0.9
(Z)-8-Heptadecene	11,000 \pm 5,100	37.0 \pm 5.1
Heptadecane	1,500 \pm 740	5.1 \pm 0.9
Unknown T ₁	230 \pm 190	0.7 \pm 0.4
Hexadecanal	440 \pm 420	1.4 \pm 1.1
Unknown A	180 \pm 100	0.7 \pm 0.4
(Z)-9-Nonadecene	1,300 \pm 980	4.7 \pm 3.5
Nonadecane	80 \pm 60	0.3 \pm 0.1
(Z)-9-Octadecenal	2,000 \pm 1,900	6.3 \pm 3.5
Octadecanal	190 \pm 130	1.0 \pm 0.7
Unknown T ₂	570 \pm 580	1.6 \pm 1.0
Geranylcitronellol (T ₃)	2,300 \pm 2,500	5.9 \pm 3.6
(Z)-11-Eicosenal	2,000 \pm 1,600	5.9 \pm 3.4
Tricosane	300 \pm 180	1.0 \pm 0.7
Tetracosane	110 \pm 50	0.3 \pm 0.1
Pentacosane	870 \pm 710	2.9 \pm 1.0
Total	30,000 \pm 16,000	

109 (5), 95 (11), 81 (26), 69 (100), 55 (20), 43 (18), 41 (61)] and T₂ [t_r 31.7 min, KI 2050, M⁺ absent, 247 (0.3), 221 (0.4), 203 (0.5), 161 (1), 147 (1), 136 (6), 123 (7), 121 (5), 109 (9), 95 (10), 81 (33), 69 (100), 55 (19), 43 (14), 41 (64)] are present. The unknown compound A is found in this species as well as in *M. gulosa*. The major components are again heptadecene (37%) and pentadecane (15%).

The positions of the double bonds were determined in the unsaturated components by converting them into their α,β -di(methylthio)ethers and submitting these to GC-MS (Francis and Veland, 1981). Each adduct gives two intense peaks that indicate the location of the double bond in the parent molecule. Heptadecene gave an adduct with prominent ions of m/z 332(M⁺), 173 [CH₃(CH₂)₇CH=SCH₃]⁺ and 159 [CH₃(CH₂)₆CH=SCH₃]⁺, indicating 8-heptadecene. This is the same position as in *M. gulosa* (Cavill and Williams,

1967). The other double-bond positions are indicated in Table 2. The retention times of the alkenes correspond to the *Z*-isomers of synthetic standards.

M. pilosula. The Dufour gland contents of *M. pilosula* are very different from the other two *Myrmecia* species examined (Figure 3, Table 3). The components are all oxygenated compounds. Long-chain acetates (C_{12} – C_{20}) predominate and the remaining compounds are long-chain aldehydes and propionates.

Individuals show a large variation in the amounts of each component present, but considering the proportions, as before, they show a smaller variance. Differences in the glandular contents of the samples from the two different sites were observed. The two major components of the Tasmanian samples were always hexadecyl acetate followed by octadecenyl acetate. However, the major component of the New South Wales samples was eicosenyl acetate for four

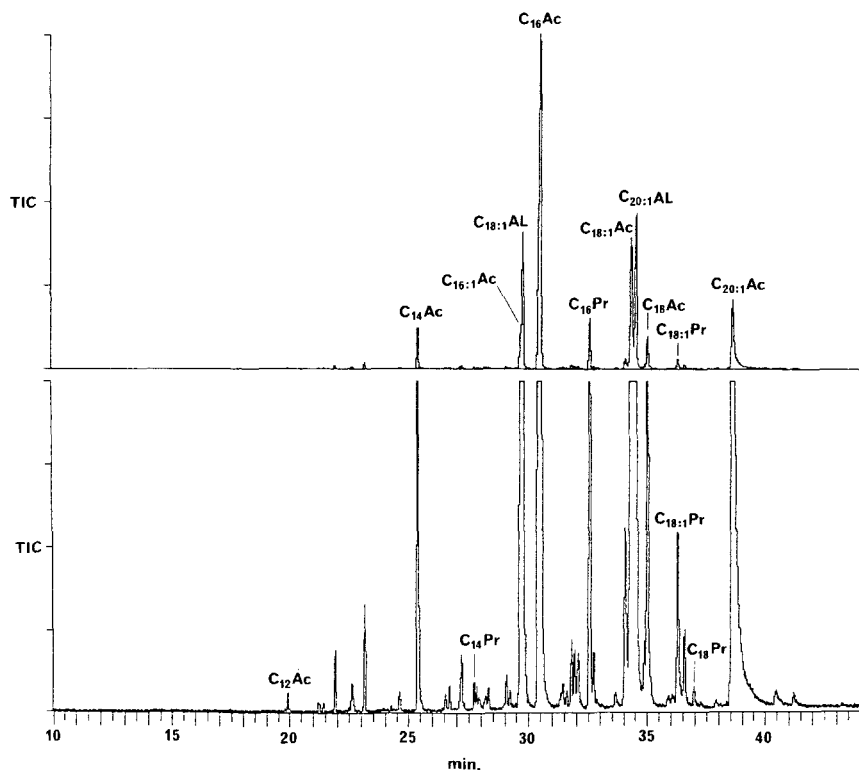


FIG. 3. Total ion chromatograms from the GC-MS analysis of the Dufour gland of a *Myrmecia pilosula* worker. Symbols indicate identification, e.g., $C_{16}Ac$ is hexadecyl acetate, $C_{18:1}AL$ is octadecenyl, $C_{16}Pr$ is hexadecyl propionate. Conditions as in Figure 1.

TABLE 3. QUANTIFICATION OF MAJOR COMPONENTS OF DUFOUR GLANDS OF WORKERS OF *Myrmecia pilosula*^a

Compound	Total		New South Wales		Tasmania	
	ng/ant ± SD	% ± SD	ng/ant ± SD	% ± SD	ng/ant ± SD	% ± SD
Tetradecyl acetate	490 ± 580	3.0 ± 2.4	190 ± 110	1.7 ± 0.8	550 ± 320	3.4 ± 1.1
Hexadecenyl acetate	690 ± 350	4.8 ± 1.1	440 ± 230	4.3 ± 1.0	900 ± 240	5.4 ± 0.9
Octadecenal	1,090 ± 910	7.1 ± 3.0	590 ± 300	5.7 ± 2.1	1,340 ± 660	7.7 ± 2.7
Hexadecyl acetate	3,730 ± 1,940	26.0 ± 7.0	2,400 ± 1,470	23.2 ± 9.0	4,930 ± 810	29.4 ± 2.7
Hexadecyl propionate	360 ± 160	3.0 ± 1.9	380 ± 180	4.4 ± 2.0	300 ± 70	1.8 ± 0.3
(Z)-9-Octadecenyl acetate	2,700 ± 1,400	19.0 ± 5.3	1,830 ± 970	17.4 ± 4.8	3,700 ± 1,200	21.7 ± 5.1
(Z)-11-Eicosenal	1,700 ± 1,000	12.0 ± 6.4	1,700 ± 1,170	15.0 ± 7.7	1,430 ± 480	8.5 ± 2.9
Octadecyl acetate	200 ± 90	1.4 ± 0.7	250 ± 110	1.9 ± 0.7	180 ± 70	1.1 ± 0.3
Octadecyl propionate	200 ± 50	1.3 ± 0.3	180 ± 60	1.4 ± 0.4	220 ± 40	1.3 ± 0.3
(Z)-11-Eicosenyl acetate	1,900 ± 1,100	14.0 ± 8.4	2,180 ± 1,350	20.1 ± 8.5	1,330 ± 230	8.0 ± 1.6
Total	13,000 ± 6,100		10,000 ± 4,000		15,800 ± 2,800	

^a Mean values and sample standard deviations for total sample (15 individuals), New South Wales (7), and Tasmania (8).

individuals, hexadecyl acetate for two, and eicosenal for another. The average proportion of octadecenyl acetate and eicosenal are approximately equal in the New South Wales sample, whereas the proportion of octadecenyl acetate is much higher than that of eicosenal in the Tasmanian samples.

The position of unsaturation of the major components was determined by GC-MS of their di(methylthio)ethers (Buser et al., 1983). The adduct of octadecenyl acetate gave prominent ions of m/z 404 (M^+), 231 $[\text{CH}_3\text{COO}(\text{CH}_2)_8\text{CH}=\text{SCH}_3]^+$ and 173 $[\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{SCH}_3]^+$, and eicosenyl acetate gave derivative ions of 432 (M^+), 259 $[\text{CH}_3\text{COO}(\text{CH}_2)_{10}\text{CH}=\text{SCH}_3]^+$ and 173 $[\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{SCH}_3]^+$. This identifies the compounds as 9-octadecenyl acetate and 11-eicosenyl acetate. The fragment ions of the eicosenal derivative, m/z 215 and 173, could arise from either 8- or 11-eicosenal. Therefore, a sequence of two nano-scale reactions was performed in which the eicosenal was reduced to eicosenol by sodium borohydride and then converted to the di(methylthio)ether. This adduct gave fragment ions at m/z 217 and 173, identifying the original compound as 11-eicosenal. Although micro-scale reactions of this kind have been used frequently by us and others (Attygalle and Morgan, 1988), we believe this is the first time a sequence of two reactions has been carried out on material at this small scale. The retention times of 9-octadecenyl acetate and its di(methylthio)ether correspond to those of oleyl acetate and its di(methylthio)ether and were shorter than those of the corresponding compounds with a *trans*-double bond, elaidyl acetate and its di(methylthio)ether. Therefore the Dufour gland component is (*Z*)-9-octadecenyl acetate, the product which would be expected as derived from oleic acid. The necessary synthetic compounds were not available for eicosenyl acetate and eicosenal, but these too may be presumed to have the *Z* configuration, and arise from (*Z*)-11-eicosenoic acid.

The amounts of propionates present generally mirrored the relative proportions of the corresponding acetates, but at less than a tenth of the amount of the acetates. Numerous minor components were observed, most of which are thought to be branched, saturated and unsaturated acetates, due to characteristic ions of m/z 43 and 61 in their mass spectra, although many of these remain to be fully identified.

In some of the samples analyzed by GC-MS, the minor components are present in relatively large amounts, although still much less than the four major components. In these samples, traces (< 10 ng) of linear hydrocarbons (heptadecene, nonadecene, hexadecene, octadecene, pentadecane, heptadecane, and hexadecane) were identified. A trace (< 5 ng) of more volatile acetates, including decyl acetate and nonyl acetate, was observed. A total of 80 components was detected.

DISCUSSION

The investigation of the Dufour gland contents of three species of *Myrmecia* by GC and GC-MS, using the solid injection technique of Morgan and Wadhams (1972), showed species-specific mixtures of hydrocarbons and oxygenated compounds.

M. gulosa has been examined before (Cavill and Williams, 1967), when six components were identified: (*Z*)-8-heptadecene (62%), pentadecane (17%), heptadecane (4%), hexadecene, tetradecane, and hexadecane. A further six components were not identified. The present study has identified 27 components, and the proportions of the major components are in good agreement with the published results, since heptadecene and heptadecadiene would have co-eluted in the earlier study so that the figure of 62% represents the total of these two compounds, which in the present work is 57.5%.

Robertson (1971) studied the pheromones involved in aggressive behavior in *M. gulosa*. Such pheromones are found in the rectal secretion, the mandibular glands, and the Dufour gland. An alarm pheromone, acting as an activation stimulus was found in the natural stinging secretions of the workers and is produced in the Dufour gland. Hydrocarbons tested for this effect gave a very weak response, and it was suggested by Robertson that the pheromone was a minor component not yet isolated. Three compounds that are not linear hydrocarbons were observed in this work among the minor components: (*Z*)-11-eicosenal, geranyl citronellol (3,7,11,15-tetramethylhexadeca-6,10,14-trien-1-ol, T₃ in Figure 1) and an unknown unsaturated compound A. Unsaturated aldehydes and terpenes are used as alarm pheromones by some species of ant, such as monoterpenes by *Myrmecaria eumenoides* and hexanal and 2-butyl-2-octenal in the complex alarm system of *Oecophylla longinoda* (Bradshaw and House, 1984). Aphids also use sesquiterpenes as alarm pheromones (Nault and Phelan, 1984). The collection or synthesis of these three minor components is now required for bioassay.

The Dufour gland secretion of *M. nigriceps* (Figure 2) is very similar to that of *M. gulosa* (Figure 1), except that more aldehydes and terpenes are present. Cavill and Williams (1967) noted that the Dufour gland secretion of *M. tricolor* (Mayr) workers showed a similar pattern of hydrocarbons to that of *M. gulosa*.

The composition of the Dufour gland contents of *M. pilosula* (Figure 3) is very different from the other species studied (Table 4), with acetates, aldehydes, and propionates being the only components. This species was collected from two different sites: in Tasmania and New South Wales. Differences were observed in the relative proportions of the major components, suggesting a geographical variation in the chemical composition of the Dufour gland of this

TABLE 4. COMPARISON OF PERCENTAGE COMPOSITION OF MAJOR COMPONENTS OF DUFOUR GLAND SECRETIONS OF THREE *Myrmecia* SPECIES AND *Nothomyrmecia macrops*

Compound	<i>Nothomyrmecia macrops</i> ^a	<i>Myrmecia gulosa</i>	<i>Myrmecia nigriceps</i>	<i>Myrmecia pilosula</i>
Pentadecane	12	17	15	
Hexadecene	1	2	1	
Heptadecadiene	3	7	3	
Heptadecene	66	50	37	
Heptadecane	5	7	5	
Nonadecene	3	2	5	
Tricosene	6			
Octadecenal			6	7
Eicosenal		3	6	12
Hexadecyl acetate	t ^b			26
Hexadecenyl acetate	t			5
Octadecenyl acetate	t			19
Eicosenyl acetate	t			14
Geranylcitronellol		6	6	

^a Billen et al. (1988).

^b t = trace.

species. There is recent karyotypic and morphological evidence for several sibling species centered on *M. pilosula* (Crosland et al., 1988). The Dufour gland composition may reflect this.

Acetates and other oxygenated compounds are found with hydrocarbons in the Dufour glands of some formicine ants (Attygalle and Morgan, 1984), but this is the first observation in ant secretions of octadecenyl acetate, eicosenyl acetate, eicosenal, and geranylcitronellol. They have all been found in other aculeate Hymenoptera: octadecenyl acetate in the labial glands of male *Bombus muscorum* (Apidae), eicosenyl acetate and eicosenal in the Dufour glands of several *Perdita* species (Adrenidae), and geranylcitronellol in the labial gland secretions of three *Bombus* species and *Psithyrus rupestris* (Apidae) (Wheeler and Duffield, 1988).

While the size of the samples used here is smaller than we would prefer to use, the consistency of composition among individuals within samples, and the similarity of results obtained for *M. gulosa* between ourselves and Cavill and Williams (1967), make some tentative conclusions possible. There is some similarity between *M. pilosula* and the others, in that they all contain eicosenal, and also, in several glands of *M. pilosula*, there were traces of some of the

hydrocarbons found in the other species. However, the Dufour gland secretions indicate two distinct groups (Table 4). This reflects morphological considerations. Greenslade (1979) describes *Myrmecia* in terms of two groups: the "inch" or "bulldog" ants and the "jumpers." *M. pilosula* is classified in the latter group, and the other species studied are placed in the former. These groups have also been described as two separate genera, *Myrmecia* and *Promyrmecia* (Clark, 1951).

These *Myrmecia* ants are very large and that is reflected in the size of their Dufour glands. Those of *M. gulosa* (25 μg mean total contents, body length 15–20 mm) and *M. nigriceps* (30 μg , body length 20–25 mm) are the largest we have encountered in the examination of many species of ant. The glands from *M. pilosula* are proportionally smaller (14 μg , body length 12 mm), as are those of *Nothomyrmecia macrops* (6.5 μg , body length 10 mm) (Billen et al., 1988).

The Dufour gland contents reflect the relationships among species, in those of *Myrmecia* studied so far. This has been observed in a number of ant genera. For example, *Tetramorium impurum* and *T. caespitum* are morphologically very similar and have very similar Dufour gland contents, whereas *T. semilaeve* is different in both respects (Billen et al., 1986).

Nothomyrmecia macrops Clark is regarded as the most primitive living ant (Taylor, 1978) and has been placed in a subfamily on its own. Its Dufour gland contents have recently been investigated (Billen et al., 1988) and showed great similarity with *M. gulosa* and *M. nigriceps* (Table 4). *N. macrops* also contains trace amounts of acetates (hexadecyl, octadecenyl, octadecyl, and eicosenyl) and aldehydes (octadecanal and hexadecanal), indicating similar biosynthetic capabilities to those of *M. pilosula* as well. Also "unknown I" of *N. macrops* is found as a trace component of *M. pilosula*.

Nothomyrmecia is placed in a separate subfamily mainly because of anatomical differences of the petiole structure (Taylor, 1978). However, *Nothomyrmecia* shares many characteristics with *Myrmecia*, and the chemical evidence of the Dufour gland secretion may indicate a closer relationship than previously thought. Further work is required on other *Myrmecia* species to see if this chemotaxonomic indicator does separate the genus into two distinct groups, and examination of further colonies of *M. pilosula* should show whether there is a consistent difference between the species in mainland Australia and Tasmania.

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ODOR OF THE MUSKOX A Preliminary Investigation

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Abstract—The behavior of captive male muskoxen was observed closely during their characteristic superiority display, the anatomy of the preputial region was studied in two adults and three calves, and preputial washings and preorbital gland secretion were subjected to gas chromatography and mass spectroscopy. During the superiority display, the prepuce was everted to form a pendulous tube tipped with a fringe of matted hair. Owing to the movement of the animal, the urine that dribbled from the preputial opening was liberally applied to the long guard hairs of the belly. The superiority display was almost exclusively confined to dominant males and apparently accounted for their odor. In the quiescent state, the hair seen around the preputial opening was drawn inside and formed an 8 cm-wide band on the lining of the prepuce. The preputial washings contained large amounts of benzoic acid and *p*-cresol. The infraorbital gland secretion contained cholesterol, benzaldehyde, and a homologous series of saturated γ -lactones ranging from 8 to 12 carbons. The latter compounds and the natural secretion smell similar to the human nose.

Key Words—Muskox, *Ovibos moschatus*, odor, preorbital gland, prepuce, rut, urine, behavior, gas chromatography, mass spectroscopy, lactone.

INTRODUCTION

The muskox has two particularly noticeable odors, the strong, rank smell of the rutting male, to which the species probably owes its name (Allen, 1913), and the light, sweetish, ethereal smell of the preorbital gland secretion. The origin

of the rutting odor has never been fully explained, although Teal (1959) recognized that it was associated with urine and was most noticeable in dominant bulls. An analysis of smegma (Teal, 1961, cited by Tener, 1965) indicated that cinnamaldehyde might be an important constituent.

The preorbital glands are well developed in muskoxen (Brinkman, 1911, cited by Schaffer, 1940; Lönnberg, 1900; Sack and Ballantyne, 1965), and they are used extensively for self-marking and scent marking in agonistic situations; this occurs during encounters between males and in both sexes when they are alarmed by predators or human activity (Gray, 1987; Gray et al., 1988). A preliminary analysis of preorbital gland tissue from a male muskox revealed the presence of cholesterol, cholesterol esters, and triglycerides but no volatile components were identified (M. Benn, footnote in Gray, 1987).

The muskox lacks discrete scent glands in other parts of the body (Schaffer, 1940), although apocrine sweat glands are widely distributed (Flood et al., 1988). There has been some confusion about the existence of interdigital glands in muskoxen (Haltenorth, 1963); our observations (unpublished) confirm Lönnberg's (1900) opinion that they are absent.

The object of the present study was to determine the source of the rutting odor and to make a preliminary investigation of the volatiles associated with the preorbital gland and the preputial region.

METHODS AND MATERIALS

Animals. The animals used in the investigation were from the research herd of muskoxen at the Western College of Veterinary Medicine, University of Saskatchewan. The herd originated from 13 hand-reared calves that were captured on Banks Island, Northwest Territories, at about 2 weeks of age in 1982 (Flood et al., 1984). At the time of sample collection, it consisted of six 3-year-old females, four entire males (two 3-year-olds and two juveniles) and four 3-year-old castrates. They were kept in a series of interconnecting pens totalling 1.5 hectares. They had free access to brome/alfalfa hay and water and were given 1.5 kg of supplemented oat pellets daily. The animals were thoroughly accustomed to human contact and could be handled without showing signs of distress.

General Observations. Day-to-day work with the research herd took 2–10 hr daily and provided a good opportunity for behavioral study. Relevant observations are reported in the results. We also report pertinent information on the anatomy and histology of the preputial region obtained from two adults and three calves that either died or were killed for various reasons.

Sample Collection from Preorbital Gland. Preorbital gland secretion was collected by placing pledgets of fine glass wool over the gland openings and

repeatedly squeezing and wiping the area. The glass wool was cleaned with methylene dichloride (CH_2Cl_2) before use and the fingers were wiped with CH_2Cl_2 before handling the glass wool. On June 27, secretion was collected from the entire herd and combined to form a single pooled sample.

Sample Collection from Prepuce. Preputial odorants were collected by instilling 60 ml of distilled water into the preputial cavity with a syringe, closing the preputial opening with finger and thumb, and massaging the preputial region for a few seconds. The contents of the preputial cavity were then collected in a wide-mouthed glass jar; this sometimes required further massaging of the prepuce. Washes were collected from all the males on June 27 and combined to provide a single sample, which was stored at -20°C until analyzed.

Extraction of Preorbital Gland Secretion. The glass wool pledgets were extracted with redistilled CH_2Cl_2 (BDH, Omnisolve, glass distilled, 3×125 ml). The combined extracts were evaporated in a rotary evaporator at 30°C and the residue redissolved in 1 ml CH_2Cl_2 prior to gas chromatography.

Extraction of Preputial Washes. Following acidification (pH 1, 10% HCl), the washes were extracted separately with redistilled CH_2Cl_2 (3×50 ml). The extract was dried (anhydrous sodium sulfate) and filtered, then treated as above.

Analysis. The preorbital and preputial extracts were treated in the same way. Gas chromatography was performed on a Varian 3700 instrument with a split injector and flame ionization detector. A DB5 column ($30 \text{ m} \times 0.32 \text{ mm ID}$) was used with helium as the carrier gas at a flow rate of $85 \text{ cm}^3/\text{sec}$. The samples were introduced at 50°C , then the oven temperature was increased at $5^\circ\text{C}/\text{min}$ to 200°C . Mass spectra were obtained on a Finnigan 4000 instrument linked to a model 2300 Inco Data Acquisition system. A DB5 column ($60 \text{ m} \times 0.32 \text{ mm ID}$) was used with a splitless injector and helium as the carrier gas at a flow rate of $45 \text{ cm}^3/\text{sec}$. The oven temperature was raised rapidly from 40°C to 90°C , then at $4^\circ\text{C}/\text{min}$ to 300°C . An electron impact of 70 eV was used scanning from mass 43 to mass 600 every second. Chemical ionization spectra were also obtained using methane as the reactant gas.

RESULTS

General. The characteristic superiority display known as "head-tilting" (Gray, 1987) was seen when the males entered their first rutting season: a bull showing this behavior walks slowly past his rival with his head tilted to display the horn boss; in doing so he uses a curious stiff-legged gait that emphasizes the height of the shoulder. Close observation revealed that during this process the prepuce was everted to form a pendulous tube about 12 cm in length tipped with a fringe of matted hair (Figure 1). The preputial tube swung about during the display and urine dribbled from its opening. As a result, the abdominal

portion of the skirt (the long, flowing, outer hairs that give the animal its characteristic appearance) became soaked with urine. This was clearly responsible for the rutting odor.

In the quiescent state the preputial tube was retracted to the level of the abdominal wall as in other ruminants and the hair previously visible at its tip was drawn inside. Dissection of the prepuce of a 3.5-year-old bull and a 4-year-old castrate showed that about 8 cm of the prepuce was lined with fine, crimped hair that was up to 3 cm in length. It covered the entire circumference of the preputial cavity and was encrusted with pale caseous particles (Figure 2). Histological examination of the prepuce of three calves less than 2 weeks old showed that the hairs were present at that age and that their follicles were directed proximally in the more distal part of the prepuce and distally in the deeper part. Sebaceous glands were associated with the hair follicles, but no sweat glands could be found (Figure 3).

Analysis of Preorbital Gland Secretion. Analysis of the methylene chloride extract of the pooled sample by gas chromatography–mass spectrometry allowed

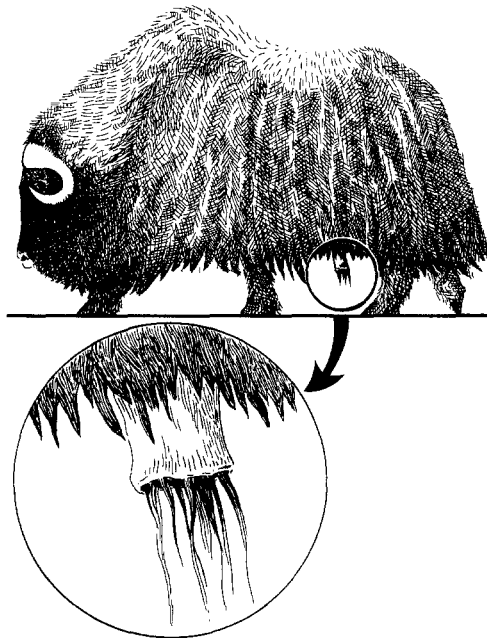


FIG. 1. An adult male muskox with the preputial tube extended and the tuft of hair exposed at its tip. The coat has been trimmed in the area within the circle so that the prepuce can be seen. The inset shows the extended preputial tube in detail. (Composite drawing made from several photographs.)

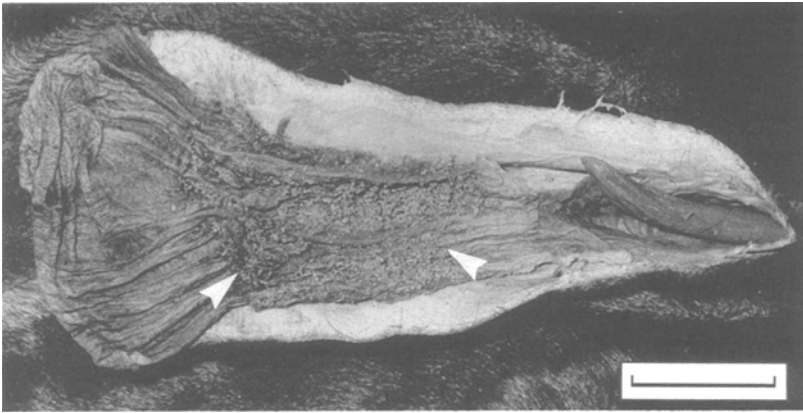


FIG. 2. The preputial cavity of a 3.5-year-old muskox opened longitudinally from the ventral side. The tip of the penis is visible on the right and the preputial opening is on the left. The hair-covered zone of the preputial lining is visible between the arrows. (Scale bar = 5 cm.)

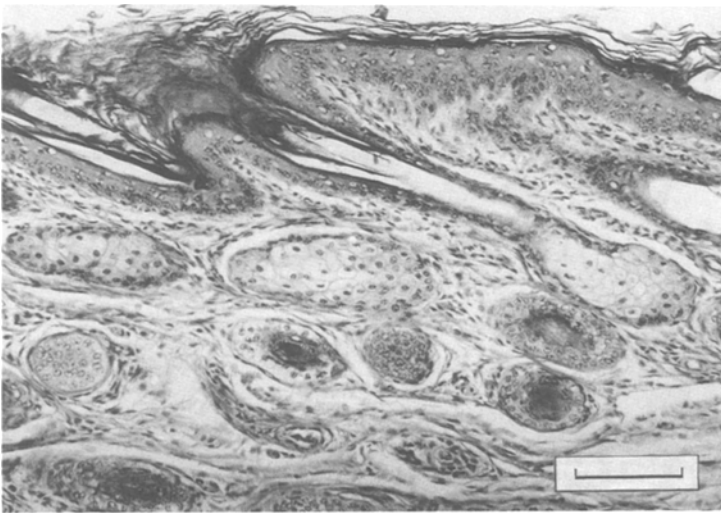


FIG. 3. Longitudinal section of the preputial skin in the haired zone from a 1-week-old calf. The lumen of the prepuce is at the top. Parts of hair shafts and several follicles are visible and these are associated with sebaceous glands. Hematoxylin and eosin. (Scale bar = 100 μ m.)

positive identification of a number of components of the mixture. Assignments of structures were made on the basis of the mass spectral fragmentation patterns observed in both the electron impact and chemical ionization modes, as well as by coelution of authentic standards on gas chromatography.

Figure 4 shows the reconstructed ion chromatogram of the preorbital extract. Cholesterol and benzaldehyde were prominent components of the mixture together with a homologous series of straight-chain, saturated γ -lactones ranging from $C_8H_{14}O_2$ to $C_{12}H_{22}O_2$. The 10-carbon lactone was present in the greatest concentration. The lactones were readily identified in the electron impact mass spectrum by a common lactone fragment (base peak m/z 85), which resulted from cleavage at the ring. The chain lengths were obtained from the chemical ionization experiments and the structural assignments were confirmed by coinjection with commercially available (Aldrich) eight- and 10-carbon γ -lactones. There was a minor peak that eluted ahead of the 12-carbon lactone that also gave a base peak of m/z 85 and a molecular ion at 196 corresponding to a monounsaturated γ -lactone.

Analysis of Preputial Washes. The major components of the CH_2Cl_2 extract of the pooled preputial washes were benzoic acid and *p*-cresol. These assignments were confirmed by nuclear mass resonance. A series of saturated straight-chain hydrocarbons ranging from $C_{22}H_{46}$ to $C_{32}H_{66}$ was present, the C_{24} homolog being the most prominent. In addition, there was a series of unidentified compounds with longer retention times than cholesterol and with molecular weights and fragmentation patterns similar to those of steroids.

DISCUSSION

Rutting Odor. Self-marking with urine is common in ruminants during the rut. Urine may be applied directly to the neck and beard (goats; Coblenz, 1976), belly (red deer; Lincoln, 1971), or legs (blacktailed deer; Müller-Schwarze et al., 1978), or it may be applied indirectly as in the bison (McHugh, 1958) and moose (Geist, 1963), which wallow in urine voided onto the ground, and the camel, which urinates on its tail, then uses this to soak the hind quarters (Wemmer and Murtaugh, 1980). The method of urine self-marking employed by the muskox is apparently unique, and the presence of hair on the inner surface of the prepuce, which is equally unusual, seems to be an anatomical correlate of the urine sprinkling behavior. In the female muskox, a homologous patch of hair is present on the ventral aspect of the vestibule.

Teal's (1959) view that the strong rutting odor is most obvious in the dominant male is supported by our own observations on the captive animals in Saskatchewan. The superiority display which, among other things, leads to the application of urine to the belly hair is also largely a prerogative of the dominant

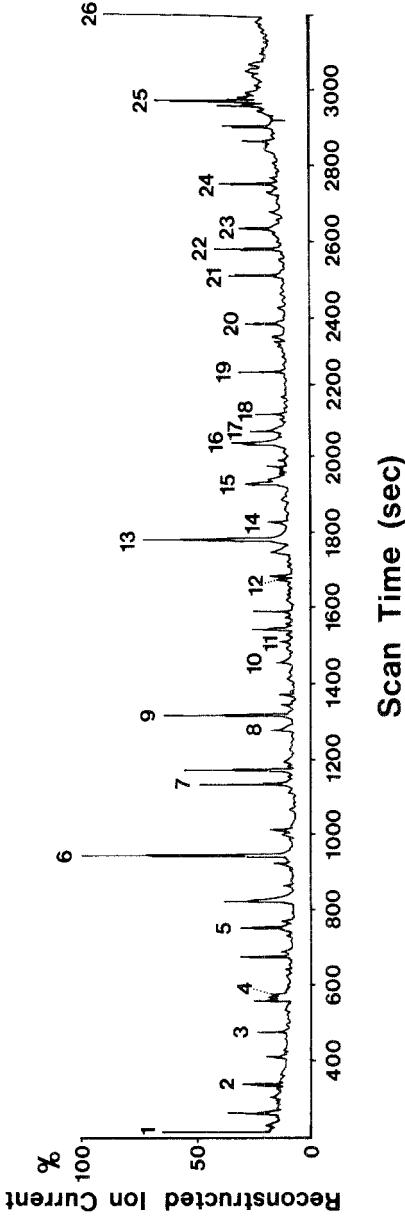


FIG. 4. The reconstructed ion chromatogram of an extract of pooled preorbital gland secretion. The numbered peaks have been identified as follows: (1) benzaldehyde, (2) nonenol, (3) decanol, (4) $C_8H_{14}O_2$, (5) $C_9H_{16}O_2$, (6) $C_{10}H_{18}O_2$, (7) $C_{11}H_{20}O_2$, (8) a monounsaturated γ -lactone, (9) $C_{12}H_{22}O_2$, (10) myristic acid, (11) $C_{16}H_{34}$, (12) $C_{19}H_{40}$, (13) palmitic acid, (14) $C_{20}H_{42}$, (15) $C_{21}H_{44}$, (16) oleic acid, (17) stearic acid, (18) $C_{22}H_{46}$, (19) $C_{23}H_{48}$, (20) $C_{24}H_{50}$, (21) $C_{25}H_{52}$, (22) phthalate, (23) $C_{26}H_{54}$, (24) $C_{27}H_{56}$, (25) $C_{29}H_{60}$, (26) cholesterol. The compounds shown by their formulas are straight-chain, saturated hydrocarbons or straight-chain, saturated γ -lactones. The peaks without numbers have not been identified.

male (Gray et al., 1988). It therefore seems likely that the characteristic odor of the dominant animal is due to his behavior rather than any profound metabolic change. Nonetheless, such a metabolic change cannot be excluded since changes in testosterone concentration are known to affect the volatile constituents of mammalian urine (Raymer et al., 1986; Schwende et al., 1986), and testosterone levels can influence and be influenced by social status (Bouissou, 1983). Further, it is known that mice (Jones and Nowell, 1974; Lombardi and Vandenberg, 1977), rats (Krames et al., 1969), and rabbits (Bell, 1984) respond differently to the urine of dominant and subordinate conspecifics.

The function of the rutting odor is unclear but a number of possibilities exist. It may advertise the presence of a dominant male and discourage interlopers. It may convey the identity of the dominant male to bulls that have challenged him previously and thereby reduce the number of fights. It may suppress reproductive activity in subordinate males (Perret and Schilling, 1987), permitting them to conserve energy. It may advance the onset of reproductive activity in the females of the herd, as does the fleece odor of the ram (Knight and Lynch, 1980) or the voice of the red deer stag (McComb, 1987), allowing the dominant male to minimize the risk that he will be deposed before the females of the herd are ready to mate. Subordinate males may be discouraged from mating with estrous females that already carry the odor of the dominant male.

The presence of phenolic compounds in the preputial washings of bull muskoxen is not unexpected since 0.5% of the content of the preputial gland of the musk deer is *p*-cresol (Sokolov et al., 1987). The preputial diverticulum of the boar is rich in *p*-cresol and also contains phenol, *o*- and *m*-methoxyphenol and *p*-ethylphenol (Patterson, 1967), and the urine-soaked belly hair of the rutting red deer contains phenol, *p*-cresol, and ethylphenol (Albone, 1984). We do not contend that the compounds identified fully account for the rutting odor of the male; clearly, some of the most volatile constituents would have been lost during concentration of the extracts and significant odorants remain unidentified. These considerations apply equally to the preorbital gland secretion considered below.

Volatiles were sought in preputial washings as opposed to urine because the odor is likely to be altered by bacterial action in the prepuce; we also believed it was important to collect some of the solids adherent to the preputial hairs. Further, the animals tolerate the procedure well; urine collection on the other hand requires prolonged restraint which causes serious distress, particularly in rutting males.

Preorbital Secretion. Perhaps the most characteristic behavior pattern of wild muskoxen is the self-marking sequence in which the preorbital region is rubbed on the outstretched foreleg (Gray, 1987; Gray et al., 1988). This results in the application of secretion to the hair of the leg and face and the release of the volatile constituents into the atmosphere. The preorbital gland may also be

rubbed on prominent objects in the environment, but this is less frequent. Use of the preorbital gland is associated with agonistic situations, but the nature of the encoded message is unknown. As in the case of rutting odor, many possibilities exist; it may betray individual identity like the inguinal glands of rabbits (Hesterman et al., 1984), and it may convey information about sex and reproductive status. The preorbital glands are much larger in males than females (Gray et al., 1988) and contain apocrine sweat glands and sebaceous components, both of which are influenced by gonadal hormones (Flood, 1985).

The presence of large amounts of cholesterol in the preorbital gland secretion is not surprising since this compound is widespread in cutaneous products; straight-chain hydrocarbons are less common, however (Albone, 1984). Benzaldehyde does not seem to be commonly identified in cutaneous secretions, but it is present in urine (Jorgenson et al., 1978) and vaginal secretion (Huggins and Preti, 1976).

The γ -lactones found in the preorbital secretion of the muskox are particularly interesting because related compounds are known to be behaviorally active in the black-tailed deer (Müller-Schwarze et al., 1978). The pure compounds also smell somewhat similar to the preorbital gland secretion. A compound that is probably isomeric with the "deer lactone," (*Z*)-4-hydroxy-6-dodecenoic acid lactone (Müller-Schwarze et al., 1978), was also found, although it was present in smaller amounts than the saturated lactones. γ -Lactones have been found in the occipital gland of the bactrian camel (Ayorinde et al., 1982), the pedal gland of the bontebok (Burger et al., 1977), and on human hair (Labows et al., 1979). We have made no attempt to determine the absolute stereochemistry of the muskox lactones.

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ELECTROANTENNOGRAM RESPONSES OF
MEDITERRANEAN FRUIT FLY, *Ceratitis*
capitata (DIPTERA: TEPHRITIDAE) TO
TRIMEDLURE AND ITS *trans* ISOMERS

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Abstract—Electroantennograms (EAGs) of unmated laboratory-reared male and female *Ceratitis capitata* (Wiedemann) were recorded in response to the attractant trimedlure [*tert*-butyl 4(and 5)-chloro-*trans*-2-methylcyclohexane-1-carboxylate] and its four *trans* isomers. For both sexes, the magnitude of the EAG response was relatively low as compared to other previously tested compounds (i.e., plant volatiles). Dosage-response curves generated for all TML isomers revealed that flies responded to increasing dosages over a relatively narrow range (two to three log steps). Responses for both sexes peaked at ca. 10 μg dose for all isomers. Antennal response in males was greatest to the C isomer followed by the B₁, A, and B₂ isomers, while responses of females were greatest for the A isomer followed by B₁, C, and B₂. Both sexes exhibited a long recovery period for the response potential to return to baseline at doses above 1 μg for all of the isomers tested, except for B₂. The low EAG sensitivity to trimedlure and the apparent EAG selectivity to the C isomer in males are discussed in relation to the known field attractancy of males to the C, A, B₁, and B₂ isomers.

Key Words—*Ceratitis capitata*, medfly, Diptera, Tephritidae, trimedlure, electroantennogram, dosage-response.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is a serious economic pest of fruit and vegetable crops throughout the world. During the past 75 years, considerable effort has been put forth to devise and improve the trapping technologies for this and other tephritid fruit fly species. Of the many compounds that have been tested as attractants for *C. capitata* (Beroza et al., 1961; Keiser et al., 1975), trimedlure (TML) [*tert*-butyl 4(and 5)-chloro-*trans*-2-methylcyclohexane-1-carboxylate] is one of the best to date and is currently used as the de facto standard for survey and detection of adult male *C. capitata*. Unlike the many pheromones or host-plant odors reported as attractants in various insects, the biological rationale as to why trimedlure, a synthetic chlorinated molecule, is attractive to male flies has remained an enigma since the serendipitous discovery of its predecessor, siglure (1-methylpropyl *trans*-6-methyl-3-cyclohexene-1-carboxylate) (Gertler et al., 1958). Trimedlure attracts both mated and unmated male *C. capitata* and has also been reported to attract virgin females in the absence of males (Nakagawa et al., 1981).

Commercial TML is composed primarily of four *trans* isomers (McGovern and Beroza, 1966) and lesser quantities of the four *cis* isomers (Leonhardt et al., 1982). The four *trans* isomers, when tested singly, elicit varying degrees of attractancy ($C \gg A > B_1 \gg B_2$), which suggests that the stereochemistry of the molecule may be an important factor (McGovern et al., 1987). Recent studies have shown the *cis* configurations of TML to be less attractive than the *trans* counterparts (McGovern et al., 1986). Although much work has been done on the isomeric chemistry of TML (McGovern and Beroza, 1966; Leonhardt et al., 1982; McGovern et al., 1986) and its attractiveness in laboratory and field tests (Beroza et al., 1961; McGovern et al., 1966, 1987), little is known about antennal sensitivity or selectivity to TML or why it elicits such a strong behavioral response, primarily in males.

Electroantennogram (EAG) recordings have been widely used to investigate detection of chemical compounds by insect antennae, but have only recently been used to study olfactory reception in tephritid fruit flies (Fein et al., 1982; Guerin et al., 1983; Van Der Pers et al., 1984; Robacker et al., 1986; Light and Jang, 1987; Light et al., 1988). These previous investigations have studied the reception of either host-plant compounds (Fein et al., 1982; Guerin et al., 1983; Light and Jang, 1987; Light et al., 1988) or identified, or putative pheromone components (Van Der Pers et al., 1984; Robacker et al., 1986; Jang et al., 1989).

The purpose of this study was to investigate the selectivity and sensitivity of adult *C. capitata* to TML and its *trans* isomers. Of special interest in this study were the questions of whether or not females possessed antennal receptors

capable of detecting these compounds and whether antennae are selectively responsive to particular isomer configurations of the molecule.

METHODS AND MATERIALS

Insects. Pupae of *C. capitata* were obtained from a laboratory mass-rearing colony maintained at the USDA, Tropical Fruit and Vegetable Research Laboratory, Honolulu, Hawaii. Upon arrival, the pupae were segregated by sex, placed in separate cages, and maintained ad libitum on sugar and water. Adult flies were tested on the second to fifth day postemergence.

Olfactory Stimuli. Commercial TML (UOP 3702) was purchased from UOP Chemical Co., East Rutherford, New Jersey, and contained the following proportions of both *trans* and *cis* isomers; *trans*: 26.9% A, 7.1% B₁, 19.7% B₂, 42.1% C; *cis*: 1.2% V, 1.3% X, 0.3% Y and W. The *trans* isomers of TML, designated as A, B₁, B₂, and C (Figure 1) according to their chromatographic retention times (McGovern and Beroza, 1966), were purified according to McGovern et al. (1987) and found to be ca. 99%+ pure by capillary GC analysis (Leonhardt et al., 1982; McGovern et al., 1986). Compounds were dissolved in spectrometric grade hexane (that was additionally distilled to 100% purity and treated with Ionox, a nonvolatile antioxidant). Serial dilutions of 100 $\mu\text{g}/\mu\text{l}$ stock solutions of the TML blend, A isomer, and B₁ isomer were made to obtain a series of logarithmic concentrations from 1×10^2 to 1×10^{-4} $\mu\text{g}/\mu\text{l}$. Compounds were delivered by pipetting 1- μl aliquots onto 1×2 -cm pieces

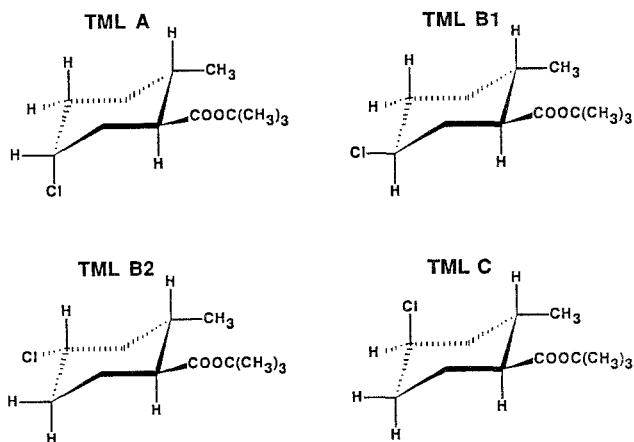


FIG. 1. Structure of *trans* trimedlure isomers.

of glass-fiber filter paper inserted into individual Pasteur pipets, which served as cartridges for the odor delivery system. Solvent was allowed to evaporate off for ca. 30 sec prior to delivery of compound over the antennae. Due to the differences in volatility of the different *trans* isomers, the less volatile isomers, B₂ and C, were tested by applying the compounds over a greater surface area of the filter paper. Based on the work by McGovern et al. (1966) on the relative volatility of these isomers, the B₂ and C isomers evaporate ca. 6.6× and 3.5× slower than the A isomer. To compensate for this difference, these compounds were diluted to obtain stock concentrations of 15.2 μg/μl and 28.6 μg/μl, respectively, and serially diluted over seven log steps. The B₂ and C isomers were applied to the filter paper at the same microgram dose as the A and B₁ isomers (i.e., 100 μg to 1 × 10⁻⁴ μg), but spread over a proportionally greater surface area (i.e., ca. 6.6× and 3.5× the size of the A isomer). This procedure "equalized" the effective volatile dose of each isomer, which was subsequently presented to the antennae. Either 6.6 μl (B₂) or 3.5 μl (C) was aliquoted onto the filter paper to achieve this effect. The diluting solvent was allowed to evaporate for 2 or 3 min before testing as determined with hexane controls using the larger volumes. These controls were found to give no higher response than the previous 1-μl volume controls.

Electrophysiological Recording Technique. Electroantennogram (EAG) techniques used in this study were identical to previously published techniques utilizing glass capillary Ag-AgCl electrodes (Light and Jang, 1987). Intact flies were immobilized by a yoke in a Plexiglas block. The recording electrode was inserted into the apical region of the terminal antennal segment, the funiculus; the indifferent electrode was positioned into the hemocoel of the cranial cavity. The signal was amplified 100× by a Grass P-16 microelectrode preamplifier and viewed on a Nicolet model 4094 digital storage oscilloscope. EAG deflections were measured from the calibrated screen and stored on floppy disks.

Odor Delivery System. The odor delivery system and stimulation apparatus were essentially the same as that described by Light (1983). A constant flow (1.0 liter/min) of charcoal-filtered and humidified compressed air was passed over the antenna through a disposable plastic pipet tip positioned ca. 1 cm from the antenna. A three-way solenoid valve diverted the purified air to the stimulus cartridge where the test odor was purged from the pipet tip and over the antenna. Stimulation was for 1 sec. For each compound, the order of presentation was always from lowest to highest dosage. A minimum of 3-4 min of clean air preceded and followed each stimulation. This time period allowed for full recovery of the antenna at the highest (100 μg) dose tested.

Experimental Procedure. EAGs were recorded from at least five individual flies of each sex for the TML commercial blend and for each of the isomers over a seven log dose range. "Control" stimulations containing either 1, 3.5,

or 6.6 μl of the hexane solvent and "standard" stimulations containing fresh cartridges of 1 μl of 1% hexan-1-ol (100% purity, Aldrich Chemical Co.) dissolved in hexane were presented between each appropriate series of compounds. EAGs were normalized to the standard stimulation by measuring the maximal amplitude of the negative deflection ($-\text{mV}$) during the stimulation period elicited by a given stimulus, subtracting the amplitude of the response to the preceding hexane control, and then dividing by the mV response to the accompanying 1% hexan-1-ol standard (from which the hexane control was also subtracted) to obtain the percent of the standard response (the standard being a 100% response in each case). This "normalization" minimized the observed variability in: (1) absolute responsiveness among preparations, (2) the time-dependent variability in antennal responsiveness, and (3) allowed for relative comparison of responses between sexes (Payne, 1975; Light, 1983; Dickens, 1984). Hexan-1-ol was chosen as a standard based on its relatively constant response as measured on many individuals in previous studies (Light and Jang, 1987; Light et al., 1988; Jang et al., 1989). The mean mV responses to standard stimulations measured for each sex in this study were $1.09 \pm 0.08 \text{ mV}$ for males and $1.14 \pm 0.11 \text{ mV}$ for females. Mean normalized TML responses were statistically compared using t tests and the nonparametric Mann-Whitney U test (Snedecor and Cochran, 1967). A "threshold" EAG response (or the minimal effective dosage needed to produce a reliably detectable response) was arbitrarily defined as the stimulus dosage at which the lower limit of the standard error to the test stimulus did not overlap with the upper limit of the standard error for the response to control (Light, 1983; Dickens, 1984). The upper limit of the standard error to the hexane control was calculated as 9.3% of standard and 5.5% of standard, respectively, for males and females.

The period of time necessary for the EAG depolarization to return to the baseline trace level for a given stimulus was defined as the recovery period. To evaluate the effects of the stimuli on antennal recovery period, the percent recovery was calculated by measuring the positive mV recovery 3 sec after the end of the stimulus, and dividing it by the maximum depolarization (see Figure 3). Comparisons of the mean percent EAG recovery at three seconds post-stimulus were made using ANOVA and Duncan's multiple-range test (Duncan, 1955).

RESULTS

Sensitivity. Dosage-response curves of male and female *C. capitata* to the commercial mixture of TML and the *trans* isomers are shown in Figure 2. In general, flies responded to increased dosages of the commercial blend and isomers A, B₁, and C over a relatively narrow dosage range (two to three log

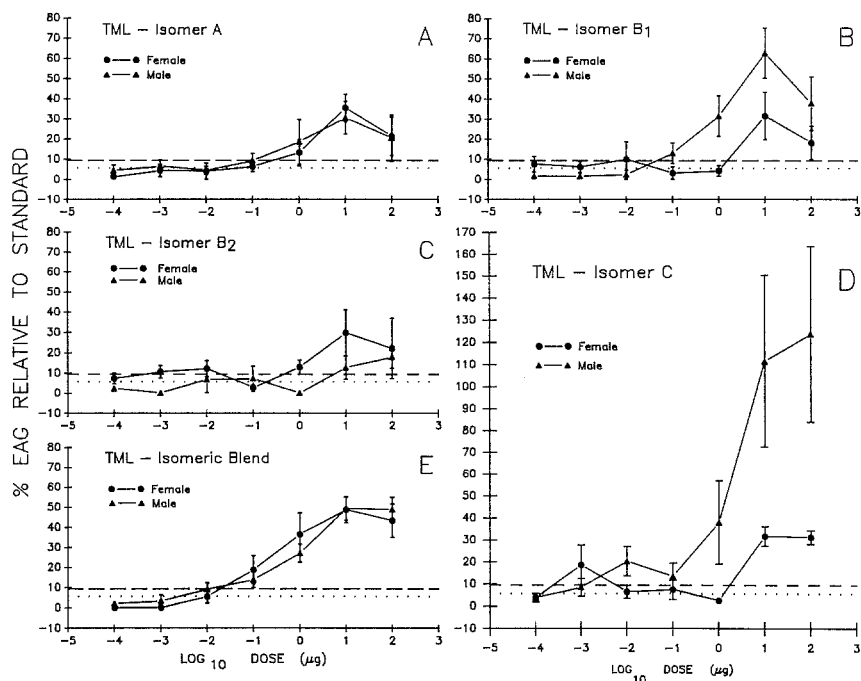


Fig. 2. Dosage–response curves of male (triangle) and female (circle) *C. capitata* to the commercial blend and individual *trans* isomers of trimedlure. Points represent the mean response \pm SE of at least five male and female flies relative to a standard (1 μ l dose of 1% hexan-1-ol), and the horizontal lines (dashes, males; dots, females) delineate threshold levels (one SE of control above zero, see Methods and Materials) above which responses are significantly greater than control ($P < 0.05$). (A) Isomer A; (B) isomer B₁; (C) isomer B₂; (D) isomer C; (E) commercial blend.

steps). Responses peaked at ca. 10 μ g for each of these isomers and for both sexes.

The antennae of both sexes showed similar EAG responses to the commercial blend (Figure 2E), with no significant differences between the sexes observed at any of the doses tested. Threshold responses appeared to occur at less than the 0.1- μ g dose for both sexes. Increased responses were evident over the dose span from above 0.01 μ g to 10 μ g, then plateaued at 100 μ g.

Isomer A of TML elicited similar EAG response curves for males and females (Figure 2A, Table 1). The threshold response occurred between 1.0 and 10 μ g for males and females. Antennae of both sexes showed a maximum response to the 10- μ g dose.

Dose–response curves to the B₁ isomer differed between the sexes in

TABLE 1. EAG THRESHOLD AND RESPONSE MAXIMA FOR MALE AND FEMALE *Ceratitis capitata*

	EAG threshold (μg)		Maximum EAG response \pm SE	
	Male	Female	Male	Female
Blend ^a (99+ %)	<0.1	<0.1	49.2 \pm 15.9	48.7 \pm 6.5
Isomer ^b A (99+ %)	>1.0	>1.0	30.5 \pm 8.2	35.6 \pm 6.8
Isomer B ₁ (97%)	>0.1	>1.0	62.9 \pm 12.6	31.6 \pm 12.2
Isomer B ₂ (99+ %)	>10.0	>10.0	17.9 \pm 5.6	29.8 \pm 11.4
Isomer C (99+ %)	>0.1	>1.0	123.8 \pm 39.9	31.7 \pm 4.6

^a Composition of commercial trimedlure (UOP 3702) *trans*: 26.9% A, 7.1% B₁, 19.7% B₂, 42, 1% C; *cis*: 1.2% V, 1.3% X, 0.3% Y and W.

^b Preparation of isomers as in McGovern et al. (1987).

threshold level, slope, and dynamic response range (Figure 2B, Table 1). Males had a lower threshold (>0.1 μg vs. >1 μg) and responses increased over a broader stimulus range than the female curve. Isomer B₁ elicited significantly greater ($P < 0.05$) responses to both the 1- μg and 10- μg doses from male than female antennae.

Neither sex responded significantly above threshold to the B₂ isomer over the majority of the dose range tested (Figure 2C). Only the females at the 10- μg dose and males at the 100- μg dose showed responses above threshold. However, relative responses were still quite low compared to the other isomers, even with compensations made for the low volatility of this isomer.

EAG responses of male *C. capitata* to the C isomer were significantly greater than those of females and were also the highest recorded among all the compounds tested (Figure 2D, Table 1). Male antennae responded above threshold at the >0.1- μg dose, while female antennae were a log step less sensitive. Maximum response was seen at the 100- μg dose for both sexes.

Selectivity. Comparing the responsiveness of both sexes to the four isomers, antennal response in males was greatest to the C isomer followed by the B₁, A, and B₂ isomers. Females exhibited maximum responses to the A and B₁ isomers followed by the C and B₂ isomers (Table 1). Differences between the sexes were most evident for the C and B₁ isomers, in both cases males having a greater response over a two to three log dose range than females (Figures 2D and B).

Recovery Period. The antennae tested exhibited a long recovery period to baseline after presentation of TML or its isomers at concentrations above 1 μg (Figure 3 and Table 2). The antennal recovery period at these higher doses was prolonged by all isomers except the B₂ isomer. Recovery periods considered

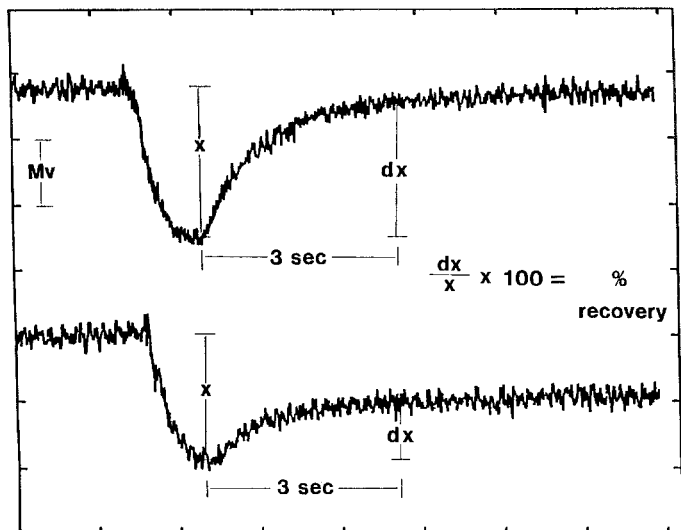


FIG. 3. Electroantennogram recording showing "normal" (hexan-1-ol standard) and "long" (isomer C, 10- μ g dose) recovery of antennal receptors. Recovery was analyzed by calculating the percent change between the maximum deflection (X) and the recovery of the stimulation trace towards baseline (dX) at 3 sec after stimulus.

"normal" were those in which the trace of the oscilloscope recording returned to the approximate baseline within ca. 3-4 sec of the end of the stimulus delivery. Stimulation traces exhibiting "long recovery" did not return to baseline within the field of view of the oscilloscope screen (ca. 10 sec). Most antennae exhibited "long recovery" at a concentration of 10 μ g, although a few were affected at lower doses. Analysis of the relative recovery rates for males after three seconds at the two highest doses tested showed significantly more long recovery for the blend, A, B₁, and C isomers than for the B₂ isomer (Table 2). Females responded similarly except for the C isomer, which was significant only at the highest dosage tested. EAGs showing long recovery did, however, return to baseline well within the minimum time period chosen between presentations of the compounds (3-4 min). The B₂ isomer and the hexan-1-ol standard exhibited "normal" recovery in all cases.

DISCUSSION

Both males and females exhibited EAG responses above threshold to the TML commercial blend and three (A, B₁, and C) of the four *trans* isomers of TML, indicating that *C. capitata* has receptor populations capable of detecting

TABLE 2. MEAN PERCENT RECOVERY TO BASELINE OF *C. capitata* ANTENNAL RESPONSES TO TRIMEDLURE AND ITS *trans* ISOMERS 3 SEC AFTER STIMULATION

Compound (μg)	Mean percent recovery after 3 sec relative to maximum ($-mV$) deflection	
	Male	Female
TML A		
0.1	94.1 \pm 4.3 abc ^a	105.2 \pm 3.1 a
1	79.5 \pm 3.2 cd	87.2 \pm 5.8 abc
10	47.3 \pm 10.2 f	45.7 \pm 9.1 efg
100	55.4 \pm 7.5 ef	44.5 \pm 9.4 efg
TML B ₁		
0.1	100.4 \pm 8.4 ab	103.1 \pm 7.6 ab
1	93.8 \pm 9.5 abc	77.7 \pm 6.3 abcd
10	58.8 \pm 7.7 ef	43.0 \pm 7.9 fg
100	56.2 \pm 2.4 ef	30.2 \pm 6.5 g
TML B ₂		
0.1	72.1 \pm 16.7 bcd	100.0 \pm 0.0 ab
1	96.2 \pm 3.8 abc	95.5 \pm 3.4 abc
10	76.1 \pm 8.0 cd	85.5 \pm 6.5 abcd
100	95.6 \pm 3.2 abc	97.1 \pm 3.2 abc
TML C		
0.1	80.3 \pm 8.8 cd	89.3 \pm 10.7 abc
1	80.7 \pm 5.4 cd	91.4 \pm 8.6 abc
10	58.3 \pm 5.0 ef	76.8 \pm 8.6 abcd
100	51.7 \pm 7.3 ef	53.7 \pm 6.6 efg
TML Blend		
0.1	87.9 \pm 8.9 abc	89.9 \pm 7.2 abc
1	88.2 \pm 5.3 abc	90.9 \pm 14.6 abc
10	52.3 \pm 5.0 ef	67.0 \pm 16.9 cdef
100	56.0 \pm 4.2 ef	56.4 \pm 12.9 defg

^aValues followed by the same letter are not significantly different from each other using Duncan's multiple-range test ($P < 0.05$). Means were calculated from at least five individuals of each sex.

these compounds. However, the relative magnitudes of these EAG responses to these compounds were relatively low compared to the hexan-1-ol standard and most plant volatiles previously tested, which elicit EAGs ranging from ca. 100% to 300% of standard at a 100- μg dose (Light et al., 1988). These plant volatiles include various monoterpenes, aliphatic esters, primary alcohols, and aldehydes; in particular, the general "green-leaf" volatiles, appear to be more potent EAG stimuli to *C. capitata* antennae. The exception was the male antennal response to the C isomer at the two highest doses, which elicited responses comparable to many of the plant volatile compounds and which elicited a significantly higher response than either the females to the same compound or

either sex's response to the A, B₁, or B₂ isomers. Significantly greater ($P < 0.05$) EAG responses between sexes were also evident in males for the B₁ isomer (at doses of 1.0 and 10 μg).

The magnitude of the EAG response is thought to be representative of the overall population size of the antennal receptors responding to a particular olfactory stimulus (Kaissling, 1971; Payne, 1975; Nagai, 1981, 1983; Mankin and Mayer, 1983; Mayer et al., 1987). Preliminary single-cell recordings (Dickens, Light, and Jang, unpublished observations) indicate the presence of cells responsive to the TML blend that are capable of detecting differences in relative doses. These TML-responsive receptor neurons were recorded from seven of the 23 cells in our preliminary sampling of sensilla distributed over various regions of the funiculus (Dickens et al., 1988). The low numbers of TML-responsive antennal receptor neurons compared to those responding to plant odors encountered in these preliminary single-cell recordings correlate with the relatively low EAG responses to most isomers, suggesting that there are low densities or small overall populations of TML receptors present. Therefore, we conclude that *C. capitata* antennae may possess small populations of receptors for the A, B₁, and B₂ isomers compared to those responsive to other semiochemicals such as selected plant volatiles. Males do, however, possess larger populations of antennal receptors responsive to the C isomer than for the other three *trans* isomers, while females may have similar numbers of receptors for the A, B₁, and C isomers. This difference suggests that males may possess specific receptors that are responding to the C isomer but are not part of the population that responds to the other isomers and may, in part, explain why the C isomer is the most active of the individual isomers in field tests. Males show a striking behavioral attraction to TML, whereas neither sex is attracted to most individual plant odors or green-leaf odors (Beroza and Green, 1963; Cunningham, unpublished observations). Primarily virgin females rather than mated females have been reported to be attracted to TML in the field (Nakagawa et al., 1981). Yet in preliminary EAG recordings to mated females (Jang, unpublished observations) we find little difference in EAG response to trimedlure or its *trans* isomers over that reported in this study to virgin females. This may indicate that other physiological states may influence perception and/or elicitation of a behavioral response to certain semiochemicals (Davis, 1984; Blaney et al., 1986).

The chemoreceptive basis for TML being a potent nonpheromonal attractant for *C. capitata* is due not only to the presence of populations of antennal receptors for these isomers, but also to the specificity and sensitivity of receptors and ultimately the CNS to specific configurations of the TML molecule. McGovern et al. (1987) suggested that the stereochemical configuration of the TML molecule was an important factor in its attractiveness based on extensive

field testing of the four *trans* isomers of TML, which showed the C isomer to be most attractive, followed by the A, B₁, and B₂ isomers, respectively. For maximal attraction in the field, they considered the axial positioning (isomers C and A) of the 4- or 5-position chlorine atom of TML to be more suitable than an equatorial conformation (isomers B₁ and B₂), and that the diequatorial position of the 1 and 2 substituents on the cyclohexane ring (*trans*) is preferable to an axial-equatorial position (*cis*). Electrophysiological evidence from this study suggests that the C and B₁ isomers are more potent antennal stimulants for males than the A or B₂ isomers at the same dose, while very little difference in EAG response to the TML isomers by the females was observed.

Although the precise significance is not known, the "long recovery" of the medfly antennal receptor neurons to high doses of TML suggests a strong interaction of the molecules with their receptors, which may be indicative of the relative "affinities" or "stickiness" of the compounds (Roelofs et al., 1969; personal communication), or slow "inactivation" of the receptor complex and/or the TML molecule (Kaisling, 1969, 1974). We found that isomers of TML that have been reported to be the most attractive (C, A, B₁) exhibited longer recovery than that of low attractancy (B₂) isomer or plant volatiles (Light et al., 1988). This difference in recovery as seen between behaviorally "active" compounds such as trimedlure and pheromones, and other more "general" odors may have a functional significance in how insects interpret chemical cues. An in-depth discussion of chemoreceptive recovery and inactivation is presented by others elsewhere (Kaisling, 1971, 1986).

We conclude from these studies that (1) both male and female *C. capitata* do possess populations of antennal receptors capable of detecting trimedlure, (2) there are differential population levels of antennal receptors responsive to different configurations of the TML molecule, and (3) based on these results, it appears that there is a relationship between differential population levels of receptors and previously published field attractancy to these same compounds. Single sensillum recordings have been initiated that may provide additional information useful in determining the precise structure-activity relationships of the TML geometric and optical isomers with antennal receptors and, perhaps, insights as to why particular isomers and/or enantiomers are more attractive than others. Future single-cell recordings on the sensitivity, specificity, and long recovery phenomena of receptor neurons from male and female *C. capitata* may reveal the mechanisms by which these isomers are detected. These studies may also reveal the neural basis of the observed behavioral differences between the sexes for both TML and host plant odors.

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FEMALE SEX PHEROMONE COMPONENTS OF *Heliothis peltigera* (LEPIDOPTERA: NOCTUIDAE)
Chemical Identification from Gland
Extracts and Male Response¹

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Abstract—Ten compounds were found in the sex pheromone glands of *Heliothis peltigera* (Schiff) and identified as tetradecenal, (Z)-9-tetradecenal, (Z)-9-tetradecenol, (Z)-9-tetradecenyl acetate, hexadecenal, (Z)-7-hexadecenal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-11-hexadecenol, and (Z)-11-hexadecenyl acetate. Behavioral tests in a wind tunnel and subsequent trapping studies conducted in the field indicated that (Z)-11-hexadecenal and (Z)-9-tetradecenal are the main pheromone components of *H. peltigera*. Addition of (Z)-11-hexadecenol to the binary blend did not enhance the capture of males of *H. peltigera*, but it decreased the number of males of the sympatric *H. armigera*. Rubber septa impregnated with a mixture of 2 mg (Z)-11-hexadecenal + 1 mg (Z)-9-tetradecenal + 0.6 mg (Z)-11-hexadecenol are recommended for monitoring *H. peltigera*.

Key Words—Sex pheromone, Lepidoptera, Noctuidae, *Heliothis peltigera*, capillary chromatography, DMDS derivatization, behavioral tests, field work.

INTRODUCTION

Heliothis peltigera (Schiff) is a polyphagous insect distributed over central and southern Europe, the Canary Islands, Asia Minor, Syria, Iraq, and Iran. It infests ornamental plants, fodder crops, medicinal crops, safflower, tobacco, grapevine, stone fruit trees, and citrus (Avidov and Harpaz, 1969; Yathom, 1971).

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In Israel it is also sometimes responsible for damage inflicted to young cotton plants and is abundant on chickpea and safflower. In order to monitor its population, particularly when adults and larvae resemble those of the sympatric *H. armigera*, work was undertaken to find attractants to be used in traps. There has been only one observation, from Sudan, on captures of *H. peltigera* in traps baited with pheromonal compounds (Outram, personal communication, in Nesbitt et al., 1979).

The purpose of the present study was to develop a specific sex pheromone blend for monitoring *H. peltigera*. To accomplish this aim, chemical identification of the pheromonal compounds in gland extracts was performed and the major components, which evoked male response in the flight tunnel, were tested in the field. The emphasis in this work was to obtain a bait, as good or better than a virgin female, that would be able to capture efficiently and specifically *H. peltigera* males. Despite the fact that 10 pheromone-like compounds were identified in the glands, there was no intention to perform a behavioral study of all the compounds. The development of sex pheromone traps for *H. peltigera* will enable monitoring and improvement of practical control procedures for this pest.

METHODS AND MATERIALS

Insects and Extracts. Females of *H. peltigera* were captured in light traps and kept for egg laying. Larvae were individually reared on a modified diet medium (Shorey and Hale, 1965). Pupae were sexed and placed in separate rooms with a dark-light regime of 10:14 hr, at $25 \pm 2^\circ\text{C}$. The insects used in the study were from this colony, which was 6–12 months old.

All ovipositors were excised from 2- to 3-day-old females, 7–8 hr into scotophase, which is the time of maximal mating in the laboratory. The ovipositors were immersed in redistilled hexane for 10–15 min, and the supernatant solution was pipetted into conical vials sealed with a Teflon-lined screw cap. Four pooled samples, each consisting of 20–30 glands, were prepared and 200 ng of Z8-13:Ac was added to each one as internal standard. The extracts were concentrated by letting them stand in a fume hood; they were stored at -18°C until use.

Chemical Identification. Capillary GC analysis was conducted using a Varian 3700 model equipped with a FID detector and a splitless injector system. A 30-m \times 0.25-mm DB 225 cyanosilicone column (J&W) was kept at 60°C for 2 min and then programmed to 150°C at $10^\circ/\text{min}$. The purge valve was opened 1 min after injection, and the helium (carrier gas) pressure was maintained at 15 psi. Quantification of peak areas was performed by a Varian 4270 electronic integrator.

Capillary GC-MS analysis was conducted on a Finnigan 5100 E1 machine at 70 eV, coupled with a Superincoss data system and interfaced with a Finnigan MAT 9610 GC. A 30-m \times 0.25-mm SE 54 (J&W) column was kept at 60°C for 2 min and then programmed from 60° to 250°C at 12°/min for pheromone components; for the dimethyl disulfide (DMDS) adducts, the column was kept at 60°C for 4 min and then programmed at 20°/min to 230°C. The column was operated in the splitless mode, the purge valve was opened 0.5 min after injection, and the helium (carrier gas) pressure was maintained at 10 psi.

DMDS adducts were prepared from several standards (Z9-14:Ald, Z9-14:Ac, Z11-16:Ald, Z11-16:OH, and Z11-16:Ac) and from a portion of the gland extracts. Samples in hexane were treated with DMDS and ethereal iodine as described by Buser et al. (1983) and Dunkelblum et al. (1985). The EI mass spectrometric analysis was conducted in the multiple ion detection (MID) mode searching for the characteristic RCHSCH_3^+ and molecular ions.

Flight-Tunnel Bioassays. The flight tunnel used ($2 \times 0.5 \times 0.5$ m) in these tests was of the same design as described by Snir et al. (1986). All tests were conducted with 4-day-old males, 6–8 hr into scotophase. The insects were kept in screen cages ($30 \times 30 \times 30$ cm) in the tunnel room. Individual males were transferred into small cylindrical cages (6 cm diameter, 6 cm high) and placed on a tripod in the center of the tunnel, approximately 20 cm from the downwind end. The tests were performed at $24 \pm 2^\circ\text{C}$, with a light intensity of 0.3 lux and a wind speed of 40–45 cm/s.

Baits were prepared by impregnating rubber septa (Maavit Products, Tel Aviv, Israel) with hexane solutions of the test compounds, and they were aged for 48 hr on a laboratory bench before use. The baits were kept in glass vials (2 cm diameter), fixed on top of a cork with a cut paper clip. The cork served as a stand for the bait in the tunnel and also enabled storage of the septa in the glass vials. They were stored in a refrigerator and kept at room temperature for 24 hr before use. Baits were positioned in the wind tunnel on a tripod approximately 20 cm from the upwind side. Ovipositor extracts from one female (1 FE), in hexane, were placed on a 1×1 -cm piece of filter paper. After evaporation of the hexane, the paper lures were fixed on a cork with a pin. Virgin females were tested as a group. Attempts to use one virgin female as a lure failed; therefore five females were placed in a screen cage and put in the tunnel. The number of calling females at a specific moment was not recorded.

Individual males were used for testing the attractancy of different blends, extracts, and live females. Four behavioral categories were recorded: (1) upwind flight (approximately half the length of the tunnel); (2) flight close to source (within 10 cm of the bait and hovering around it); (3) source contact, and (4) copulation attempts. Results were analyzed by the χ^2 2×2 test of independence using the actual number of insects and are presented as percentage of response. Only moths that responded within 2 min of release were scored.

Field Work. Tests were conducted in cotton, chickpea, and safflower fields in the Bet Shean Valley, and at Mai Ami and Bet Dagan during the 1987 and 1988 seasons. Rubber septa dispensers were loaded with hexane solutions of test compounds containing 10% of butylated hydroxytoluene (BHT) as antioxidant. The traps used consisted of a plastic ring (9 cm diameter) with a plastic roof fixed 3–5 cm above the opening; the dispenser containing the synthetic chemicals was hung under the protecting roof. A nylon bag was attached to the plastic ring to collect the trapped insects. Attractancy of virgin females was tested by hanging a small screen cage containing two virgin females under the protecting roof. Talcum powder was inserted into the nylon bag to prevent the escape of captured moths. The traps were fixed at a height of ca. 1.5 m above the ground; adjacent traps were located at least 30 m apart. Captured moths were collected and counted every one to three days. Positional bias was reduced by moving the different treatments one position whenever the traps were examined. In all experiments, three to five traps were used for each treatment. Duncan's new multiple-range test was used to analyze significant differences between treatments.

RESULTS

Chemical Identification. Capillary GC analysis of the ovipositor extracts revealed the presence of a large number of peaks (Figure 1). Comparison of the retention times with synthetic compounds enabled the tentative identification of 10 components: tetradecanal (14:Ald), (Z)-9-tetradecenal (Z9-14:Ald), (Z)-9-tetradecenol (Z9-14:OH), (Z)-9-tetradecenyl acetate (Z9-14:Ac), hexadecanal (16:Ald), (Z)-7-hexadecenal (Z7-16:Ald), (Z)-9-hexadecenal (Z9-16:Ald), (Z)-11-hexadecenal (Z11-16:Ald), (Z)-11-hexadecenol (Z11-16:OH), and (Z)-11-hexadecenyl acetate (Z11-16:Ac) (Table 1). The amount of the major component Z11-16:Ald in the pooled extracts was 23–42 ng/female. The polar DB 225 capillary column gave a fair separation of Z7-16:Ald, Z9-16:Ald, and Z11-16:Ald.

Additional information on the identity of the compounds present in the gland extracts was obtained from the GC-MS analysis on a SE 54 capillary column. The amount of some of the trace compounds, such as 14:Ald, was insufficient for a mass spectrum. Two of the minor unsaturated aldehydes, Z7-16:Ald and Z9-16:Ald eluted on the SE 54 column as one peak. The mass spectrometric data were in accordance with the proposed structures. Derivatization of a portion of the extracts with DMDS and subsequent GC-MS analysis enabled the separation of all unsaturated components. It confirmed the presence and established the position of the double bond in all these components. The mass spectrometric analysis in the MID mode enhanced the sensitivity of the

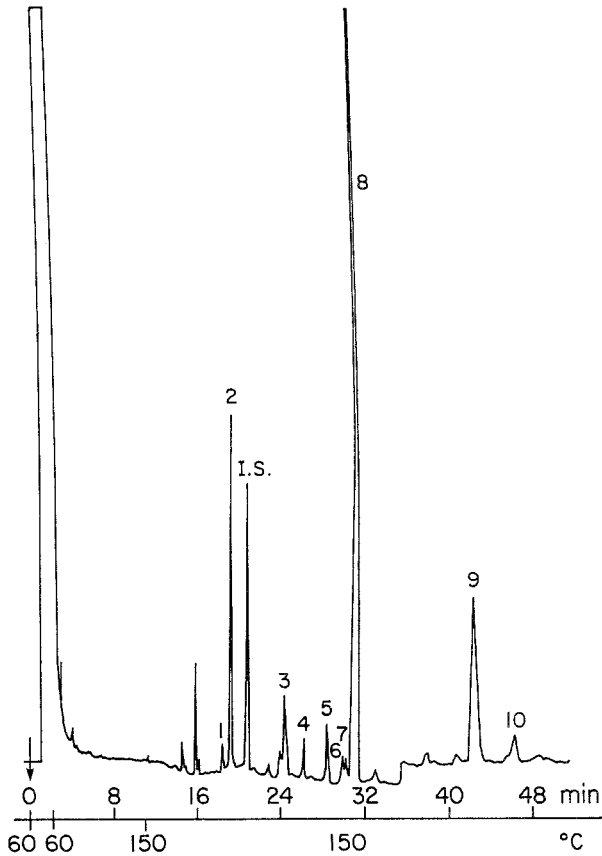


FIG. 1. Capillary GC trace of gland extracts of *Heliothis peltigera*: 1 = 14:Ald, 2 = Z9-14:Ald, 3 = Z9-14:OH, 4 = Z9-14:Ac, 5 = 16:Ald, 6 = Z7-16:Ald, 7 = Z9-16:Ald, 8 = Z11-16:Ald, 9 = Z11-16:OH, 10 = Z11-16:Ac, and I.S. = Z8-13:Ac.

analysis and enabled the detection of all the predicted DMDS adducts. The intense $RCHSCH_3^+$ ions as well as the molecular M^+ ions were diagnostic for the structure of all the adducts. The mass spectrometric data are summarized in Table 2.

Flight-Tunnel Bioassays. The flight-tunnel tests reported here concentrated on the components present in largest amounts: Z11-16:Ald, Z9-14:Ald, and Z11-16:OH. The best blends were compared with ovipositor extracts and with virgin females. In all flight tests, most males that reached the bait performed copulation attempts. This behavioral stage was readily recognized by the expo-

TABLE 1. RELATIVE PROPORTION OF COMPOUNDS IDENTIFIED FROM SEX PHEROMONE OVIPOSITORS OF *Heliothis peltigera*^a

Compound	Average (%)	Range (%)
14:Ald	0.8	0.5-1.0
Z9-14:Ald	14.6	13.0-17.5
Z9-14:OH	6.5	4.4-7.6
Z9-14:Ac	2.0	1.6-2.5
16:Ald	3.7	3.3-4.0
Z7-16:Ald	1.1	0.5-1.5
Z9-16:Ald	1.5	1.2-2.0
Z11-16:Ald	100	
Z11-16:OH	24.3	21.7-26.1
Z11-16:Ac	4.8	3.3-8.0

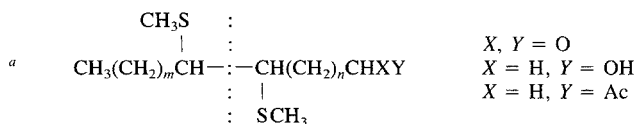
^aRelative proportions were calculated with respect to the main component Z11-16:Ald.

sure of the hairpencils and the bending of the abdomen. The flight tests preceded and served as a basis for the subsequent field tests.

In the first behavioral tests (Table 3), the activity of the two major components, Z11-16:Ald (HP1) and Z9-14:Ald (HP2), was tested individually

TABLE 2. MASS SPECTROMETRIC DATA OF DMDS DERIVATIVES OF UNSATURATED COMPOUNDS FROM PHEROMONE BLEND OF *Heliothis peltigera*^a

DMDS adduct ^b	A ⁺	B ⁺	B ⁺ - 60	M ⁺
Z9-14:Ald	117	187		304
Z9-14:OH	117	189		306
Z9-14:Ac	117	231	171	348
Z7-16:Ald	173	159		332
Z9-16:Ald	145	187		332
Z11-16:Ald	117	215		332
Z11-16:OH	117	217		334
Z11-16:Ac	117	259	199	376



A⁺ B⁺

^bPresented in the order of elution on the SE 54 column.

TABLE 3. RESPONSE (%) OF MALE *Heliothis peltigera* IN WIND TUNNEL TO PHEROMONAL BLENDS, GLAND EXTRACTS, AND LIVE FEMALES^a

Bait ^b	Components (μ g)			Number tested	Upwind flight	Close to source	Source contact	Copulation attempts
	Z11-16:Ald	Z9-14:Ald	Z11-16:OH					
HP1	2000			26	100a	8d	0c	0c
HP2		1000		33	100a	18cd	0c	0c
HP3	2000	20		24	100a	29c	8c	8c
HP4	2000	100		41	98a	73b	66ab	59ab
HP5	2000	1000		71	100a	92a	73ab	61ab
HP6	2000		600	32	100a	12cd	0c	0c
HP7	2000	1000	600	61	95a	90a	77a	74a
1 FE				26	92a	62b	46b	46b
♀ ♀ ^c				28	100a	57b	54b	54ab

^aPercentages followed by the same letter are not significantly different according to χ^2 test of independence.

^bAll baits were rubber septa impregnated with the tested compounds, except for 1 FE, which was a one gland extract on a 1-cm² filter paper.

^cFour or five females confined in a 30 × 30 × 30-cm screen cage.

and compared with that of several binary blends consisting of these two components in different ratios (HP3, HP4, HP5). Subsequently, a blend of Z11-16:Ald + Z11-16:OH (HP6) and a ternary blend of the two aldehydes and the alcohol were examined. The results showed that both Z11-16:Ald and Z9-14:Ald induced only upwind flight. Addition of varying amounts of Z9-14:Ald to Z11-16:Ald caused a significant increase in attractancy of the blends. Mixtures containing 5% of Z9-14:Ald (HP4) or 50% of Z9-14:Ald (HP5) performed similarly, with approximately 60% of the males completing the whole courtship sequence. Addition of Z11-16:OH to Z11-16:Ald (HP6) had no influence on the performance of the bait. In further tests the binary blend, HP5, was compared with a ternary blend of Z11-16:Ald + Z9-14:Ald + Z11-16:OH (HP7). A slight increase in activity was observed, but the difference was not significant. The ternary blend HP7 was as active as virgin females and more active than the one-ovipositor extract (1 FE).

Field Tests. Most of the field tests were performed during 1987 at two locations on two different crops at the beginning of the season, and later on cotton alone (Table 4). Initial results (test 1) indicated that the mixture of Z11-16:Ald + 2.5% Z9-16:Ald (HA), the two major sex pheromone components of *H. armigera* (Kehat et al., 1980), did not attract *H. peltigera* males. The addition of 5% Z9-14:Ald to this mixture (HP8) resulted in trapping of *H. peltigera* on the one hand, and in a significant decline in captures of *H. armigera* on the other. Increasing the amount of Z9-14:Ald up to 20% (HP9) sig-

TABLE 4. CAPTURES OF *Heliothis peltigera* AND *Heliothis armigera* (MEAN NUMBER OF MALES/TRAP/NIGHT)^a IN TRAPS WITH DIFFERENT PHEROMONAL BLENDS

Test ^b	Bait	Components (μ g)					Location/crop			
		Z11-16:Ald	Z9-16:Ald	Z9-14:Ald	Z11-16:OH	H. arm.	H. pelt.	H. arm.	H. pelt.	
1	HA	2000	50			Mai Ami/cotton		Mai Ami/chickpea		
	HP8	2000	50	100		22.7a	0c	57.5a	0c	
	HP9	2000	50	400		0.5b	0.1b	2.7b	0.6b	
2						0.1c	0.9a	0.1c	1.5a	
						Mai Ami/cotton		Bet Shean/cotton		
	HA	2000	50				0	21.0a	0	
	HP9	2000	50	400			1.10a	0.30b	0.60a	
	HP10	2000	50	1000			1.50a	0.01c	0.60a	
3							1.70a	0.05c	0.80a	
						Mai Ami/cotton				
	HA	2000	50			7.20a	0b	6.3a	0b	
	HP11	2000		400		1.10b	0.20a	0.14b	0.10a	
	HP5	2000		1000		0.10c	0.20a	0.02c	0.11a	
				600		0.01d	0.45a	0c	0.28a	

^a Means followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's new multiple-range test.

^b Test 1, April 27-May 15, 1987; test 2, June 6-24, 1987, catches of *H. armigera* were not recorded at Mai Ami; test 3, June 26-July 14, 1987.

TABLE 5. CAPTURES OF *Heliothis peltigera* (MEAN NUMBER OF MALES/TRAP, NIGHT)^a IN TRAPS WITH BLEND OF HP5, HP7, AND VIRGIN FEMALES IN COTTON FIELDS

Test ^b	Bait	Components (μg)			<i>H. peltigera</i>
		Z11-16:Ald	Z9-14:Ald	Z11-16:OH	
1	HP5	2000	1000		7.7a
	HP7	2000	1000	600	7.5a
2	HP5	2000	1000		10.2a
	2 ♀				4.2b

^aMeans followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's new multiple-range test.

^bTest 1, March 24-31, 1988, in Bet Shean; test 2, May 5-9, 1988 at Bet Dagan.

nificantly increased the captures of *H. peltigera* and further reduced the number of trapped *H. armigera* males (HP8 vs. HP9). Results presented in test 2 indicated that increasing the amount of Z9-14:Ald up to 50% (HP10) did not enhance captures of *H. peltigera* as compared with the addition of only 20% Z9-14:Ald (HP9) but drastically reduced captures of *H. armigera*. The minor component in HA, Z9-16:Ald, did not affect the captures of *H. peltigera*, as shown by the comparison of HP10 with HP5. Therefore, this compound was eliminated from additional field tests. In test 3, two binary blends of Z11-16:Ald and Z9-14:Ald (HP11 and HP5) were compared with a ternary blend of these components and Z11-16:OH (HP7). The results showed that all three lures captured the same numbers of *H. peltigera* males, but larger amounts of Z9-14:Ald (HP5 vs. HP11) resulted in a smaller trap catch of *H. armigera*. Addition of 30% of Z11-16:OH, which is a strong inhibitor to *H. armigera* (Kehat et al., 1980), resulted in complete elimination of males of *H. armigera* from the traps (HP5 vs. HP7).

Two field tests were performed in the spring of 1988, when the population density of *H. peltigera* was higher (Table 5). The first test again compared the efficiency of the best binary blend (HP5) with the ternary blend (HP7), and results indicated that they have the same attractancy in the field. The second test compared blend HP5 with two virgin females, and the synthetic lure attracted significantly more males than did the females.

DISCUSSION

The sex pheromones of several *Heliothis* species—*H. virescens* (Klun et al., 1980a), *H. zea* (Klun et al., 1980b), *H. armigera* (Nesbitt et al., 1979, 1980; Dunkelblum et al., 1980), *H. subflexa* (Teal et al., 1981; Klun et al.,

1982), *H. puntiger* (Rothschild et al., 1982), and *H. phloxiphaga* (Raina et al., 1986) have been investigated in recent years. For most *Heliothis* species, gland extracts were analyzed and multicomponent pheromones were found. Only in *H. virescens* was airborne pheromone from live females (Teal et al., 1986) and from gland volatiles (Pope et al., 1982) collected and analyzed. Gland volatiles were also analyzed from *H. zea* (Pope et al., 1984). In all the *Heliothis* species, the main pheromone component was Z11-16:Ald, accompanied by varying amounts of other C₁₆ aldehydes, acetates, and alcohols. Only in *H. virescens* was Z9-14:Ald found to be an essential component in addition to the C₁₆ components (Vetter and Baker, 1983; Teal et al., 1986).

Comparison of the content of the gland extract from *H. peltigera* (Table 1) with the pheromone components of the other *Heliothis* species showed that *H. peltigera* contains most of the compounds found previously in the other species. The composition of the gland extracts from *H. virescens* (Teal et al., 1986) is similar to that of *H. peltigera*, although the latter contains also the two acetates, Z9-14:Ac and Z11-16:Ac, not present in *H. virescens*. There is also some similarity to the composition of gland extracts of *H. subflexa* (Teal et al., 1981; Klun et al., 1982). However, the latter contains additional alcohols and acetates. Most important, *H. subflexa* contains relatively large amounts of Z9-16:Ald and only trace amounts of Z9-14:Ald, whereas in *H. peltigera* the opposite was found.

The gland extracts of *H. peltigera* contain a large number of pheromonal compounds, but probably only some of these compounds are emitted by the female; the rest of the components may be biosynthetic precursors and intermediates (Morse and Meighen, 1987). The different functional groups of aldehyde, alcohol, and acetate can be biosynthetically interconverted, as shown in *Choristoneura fumiferana* (Morse and Meighen, 1987) and in *H. virescens* and *H. subflexa* (Teal and Tumlinson, 1987). The presence of the three functional classes in *H. peltigera* may indicate the biosynthetic pathway of the formation of the pheromone components in the glands of this moth.

The principal goal of the present study was to identify the main pheromone components of *H. peltigera* and to develop a species-specific lure for monitoring *H. peltigera* in the presence of the sympatric species, *H. armigera*. Therefore, the major effort was directed at the identification of the compounds in the pheromone gland that are attractive to males in both the flight tunnel and field.

The flight tests were planned to find the main components that evoke maximal response from the males and eventually would be useful in the field. The tested lures were evaluated by comparing their activity with that of calling females. A relatively high load per septum, based on 2 mg of Z11-16:Ald, was used because this is the standard load of pheromones in use for monitoring other lepidopteran pests in Israel. In view of the information on the pheromone blends of other *Heliothis* species, the obvious choices to start these behavioral tests were the two most abundant aldehydic compounds, Z11-16:Ald and Z9-

14:Ald. The tests showed that these compounds initiated individually only upwind flight, but a mixture of the two evoked a strong response from the males, comparable to that of calling females. The fact that the binary blends of Z11-16:Ald + Z9-14:Ald evoked the whole courting sequence from the males was very encouraging. The alcohol Z11-16:OH, which was one of the more abundant compounds in the gland extracts of *H. peltigera*, is an inhibitor to the sympatric *H. armigera* males (Kehat et al., 1980). Therefore, it was important to evaluate the response of the *H. peltigera* males to this compound. Addition of 30% Z11-16:OH to the binary blend of aldehydes indicated that this alcohol has no inhibitory effect on *H. peltigera* males. Subsequent field tests showed that a 2:1 mixture of Z11-16:Ald and Z9-14:Ald is an efficient attractant for *H. peltigera*, better than two virgin females. The addition of Z11-16:OH did not enhance the trap catch, but produced a more species-specific lure for *H. peltigera* by reducing completely the capture of the sympatric *H. armigera* males. The minor component Z9-16:Ald, which is present in both *H. armigera* and *H. peltigera*, is an essential component in the pheromone blend of *H. armigera* (Kehat et al., 1980) but seems to have no effect on the trap catch of *H. peltigera*. Despite that fact that the present results are based on the composition of gland extracts, the data from both the flight tunnel and field tests indicated strongly that Z11-16:Ald and Z9-14:Ald are the essential pheromone components of *H. peltigera*. The alcohol Z11-16:OH might be a pheromonal component responsible for enhancement of the pheromone specificity. Therefore, the ternary blend of Z11-16:Ald + Z9-14:Ald + Z11-16:OH is recommended for monitoring *H. peltigera*.

Careful airborne collection from live females is necessary in order to determine which components, from the large pool of compounds in the gland, are emitted as pheromone components. Based on these findings, additional behavioral tests with synthetic lures emitting the same blend should be performed. At the present time, the major pheromone components have been identified and evaluated by flight and field tests. A lure has been developed that efficiently and specifically attracts *H. peltigera*, thus enabling monitoring of the pest and possibly leading to improved procedures for its control.

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CHEMICAL MIMICRY IN A PARASITOID
(HYMENOPTERA: EUCHARITIDAE) OF FIRE ANTS
(HYMENOPTERA: FORMICIDAE)

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Abstract—A wasp (*Orasema* sp.) parasitic on the fire ant, *Solenopsis invicta* Buren, develops to the adult stage within the ant colony, where wasp larvae are ectoparasitic on ant pupae. This phase of the parasite's life cycle requires a mechanism of integration into the host colony. Gas chromatographic profiles of hexane soaks of various stages of the parasite and host suggest that during development within the ant colony the parasite acquires the colony odor of the host through a passive mechanism, based on simple contact and other social interactions. No parasite-specific components were observed. After leaving the host nest as adults, the parasite biosynthesizes a parasite-specific cuticular compound, while retaining residual amounts of the host acquired components. This complicated scenario is consistent with current knowledge of nestmate recognition and the preferential treatment of ant workers to their brood.

Key Words—Parasite, fire ant, *Solenopsis invicta*, *Orasema*, Hymenoptera, Formicidae, mimicry, nestmate recognition, cuticular hydrocarbons, Eucharitidae.

INTRODUCTION

The search for natural enemies of the fire ant, *Solenopsis invicta* Buren, has revealed a number of diseases, parasites, and inquilines (Jouvenaz, 1986; Wojcik et al., 1987). One of these is a wasp, *Orasema* sp. (Hymenoptera: Chalcidoidea: Eucharitidae), that parasitizes *S. invicta* in Brazil. Species of Eucharitidae only parasitize ants (Das, 1963; Johnson et al., 1986; Williams

and Whitcomb, 1974) and have an unusual life cycle. Female wasps oviposit in plants (leaves, buds, fruit) that are frequented by ants. The planidia (newly hatched larvae) attach themselves to foraging ants and are carried to the nest (Wheeler, 1907; Johnson et al., 1986). The planidia then make their way to ant brood and complete their development as ectoparasites (Wojcik, 1986).

The apparent inability of fire ants to distinguish between wasp brood and their own brood suggested that the *Orasema* sp. mask their odor in some way to confuse the ants' nestmate-recognition mechanism. There is ample precedence for chemical mimicry among myrmecophiles and termitophiles. Adults of the myrmecophilous scarab beetle [*Martinezia duterteri* Chalumeau = *Myrmecaphodius excavatiocollis* (Blanchard)] integrates into host colonies of fire ants, *Solenopsis* spp., by passively acquiring the colony odor of the host (Vander Meer and Wojcik, 1982). Integrated beetles move unmolested among host ants and obtain food directly from workers by trophallaxis, by predation on ant brood, and by feeding on dead ants (Wojcik, 1975). Chemical mimicry also occurs in syrphid flies (*Microdon* sp.) that live in ant nests (R. Howard, USDA, Manhattan, Kansas, personal communication). In addition, it has been proposed that myrmecophilous lycaenid larvae successfully associate with their hosts through mimicry of volatile secretions produced by the host ant and its brood (Henning, 1983). The termitophilous beetle, *Trichosenius frosti* Seevers, biosynthesizes the same cuticular hydrocarbons as its host, *Reticulitermes flavipes* Kollar (Howard et al., 1980). These compounds are believed to be important in termite nestmate recognition. Three other termitophiles have been shown to have a cuticular hydrocarbon profile identical to that of their hosts (Howard et al., 1982).

We report the results of studies that compare chemical profiles of cuticular washes of an *Orasema* sp. parasite and its host during parasite development within the host colony and of adult parasites captured away from their host colony.

METHODS AND MATERIALS

Source of Parasite and Host. Colonies of *S. invicta* were collected for biocontrol screening in the vicinity of Cáceres, Mato Grosso, and Campo Grande, Mato Grosso do Sul, Brazil, in 1985 and 1986, and the ants were separated from the soil by flotation (Jouvenaz et al., 1977). In addition to the adult and immature wasps and ants collected from nest soil (Cáceres), adult male and female wasps were captured with an insect net as they swarmed over fire ant nests (Campo Grande). Queenless, miniature laboratory colonies composed of conspecific adult and immature worker ants, with and without wasp parasites (wasps were always maintained with the host colony from which they

were collected), were maintained in soil-free nests for up to one week by methods described by Banks et al. (1981). To facilitate observation, colonies were limited to fewer than 200 individuals.

Sample Preparation. Pupae and adults of both wasps and ants were placed individually and in groups in 7-ml vials that contained ca. 0.5 ml HPLC-grade *n*-hexane (Merck, Darmstadt, West Germany). The vials were capped with aluminum foil-lined lids and allowed to stand at room temperature for 45 min or 24 hr. The hexane was transferred by Pasteur pipet to 2-ml vials. The vials were loosely capped and the hexane allowed to evaporate. The cap was then tightened. Great care was taken to avoid cross-contamination of the samples. All samples were externally labeled and shielded from sunlight and foreign matter. They were hand carried to our laboratory in Gainesville, Florida, where they were reconstituted with HPLC-grade hexane (Burdick and Jackson, Muskegan, Michigan) prior to analysis by gas chromatography.

Chemical Analysis. Gas chromatographic (GC) analyses were carried out on a Varian 3700 gas chromatograph (Walnut Creek, California) equipped with a flame ionization detector and a 30-m \times 0.032-mm-ID DB-1 fused silica capillary column (J&W Scientific, Inc., Rancho Cordova, California). Helium was used as the column carrier gas, and nitrogen was used as the makeup gas. The following two oven temperature programs were used: (1) 50°C for 1 min then increased to 285°C at 5°/min and held for a total run time of 70 min; (2) 150°C for 1 min then increased to 285°C at 4°/min for total run time of 35 min. The chromatograms were printed and peak areas calculated on a Vista 401 data processor (Varian). If injection of 1 μ l of the reconstituted samples gave a weak chromatogram, then they were concentrated under a stream of nitrogen and run again. The accuracy of peak integration was checked by replotting the chromatograms with integration baselines.

Quantitative Analysis of Orasema sp. Adults. Samples of evaporated hexane soaks of individual *Orasema sp.* adults were reconstituted with hexane. Three microliters of a 0.01% hexane solution of *n*-pentacosane was added as an internal standard. The samples consisted of three replicates of each of the following: (1) males collected from the host ant nest; (2) females collected from the host ant nest; and (3) males collected from outside potential host ant nests. Gas chromatograms were obtained for each sample as described above and the data quantified using the Varian Vista data processor. The quantitative data was statistically analyzed using the Newman-Keuls test.

Mass Spectral Analysis. Mass spectra were obtained on a Hewlett-Packard 5988A GC-MS (Hewlett-Packard Co., Palo Alto, California) that was operated in the EI mode (70 eV) and equipped with an HP 9000/300 Chemstation and interfaced with an HP 5890 GC operated in the splitless mode. An HP methyl silicone fused silica capillary column (12 m \times 0.20 mm ID) was used for the chromatographic separation. The GC oven was operated at 60°C for 1 min then

to 225°C at 25°/min and finally to 300°C at 7°/min. The mass spectra of GC-separated components derived from pooled samples of *Orasema* sp. pupae and host colony pupae were compared. Comparison of the chromatograms of *Orasema* sp. pupae and adults with the corresponding host ant chromatograms was accomplished using the Newman-Keuls test, comparing, one at a time, the percentages of the five dominant peaks in the chromatograms.

RESULTS

Parasitism in fire ant colonies by *Orasema* sp. (identified as *Orasema* sp. by L. de Santis, Universidad Nacional de La Plata, La Plata, Argentina) can be common, with up to 41% of the colonies sampled containing the parasites and 1–598 wasps per colony (Wojcik et al., 1987). Wasp larvae and pupae were visually distinguishable from host ant larvae and pupae. In the disturbed conditions of isolating ants and parasites by flotation (Jouvenaz et al., 1977), worker ants carried wasp larvae and pupae around in the same way ant brood was carried. Wasp pupae were readily adopted by conspecific colonies of fire ants. No aggressive behavior toward wasp pupae was observed. One female wasp eclosed in an observation nest. She did not move about the nest, but remained immobile and was tended with the ant brood. After two days, the adult wasp was presumed dead and was removed from the nest for examination, whereupon she immediately flew away.

Gas chromatograph (GC) traces of host worker ants show the characteristic venom alkaloid pattern of *S. invicta* (Figure 1A). These piperidine alkaloids are found in large amounts (10–20 µg) in the fire ant poison sac and are released into the solvent during the hexane soak period. They are composed of 6-methylpiperidine alkaloids with 2-substituted alkyl or alkenyl side chains (Brand et al., 1972). The structures are simply defined by the chain length of the 2-position side chain and whether or not it contains a double bond; i.e., $C_{13:1}$ = 2-tridecenyl-6-methylpiperidine; see Figure 1A. In addition, Figure 1A illustrates the species-specific hydrocarbon patterns characteristic of *S. invicta* (section A of Figure 1A) (Vander Meer, 1986). These components have been identified as a series of normal, monomethyl and dimethyl branched hydrocarbons (Lok et al., 1975; Nelson et al., 1980). The patterns and peak retention times were directly compared with authentic samples of *S. invicta* venom alkaloids and cuticular hydrocarbons.

Gas chromatograms of hexane soaks from male and female *Orasema* sp. adults collected from their host net (Figure 1B and C) showed an identical qualitative hydrocarbon pattern to that of its ant host (Figure 1A) established by comparison of retention times and mass spectra of the five major components. The mass spectra were completely in accord with published spectra (Lok et al.,

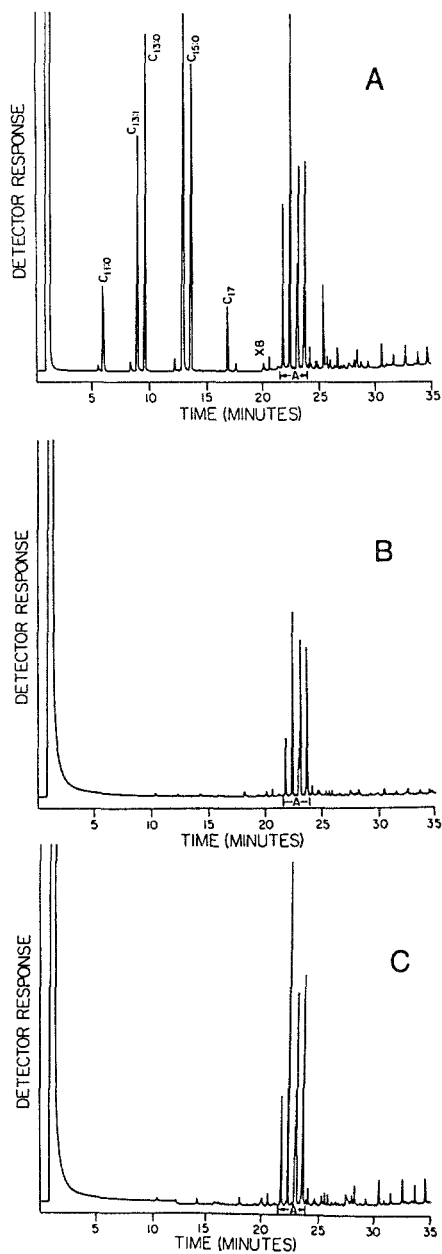


FIG. 1. Total gas chromatograph volatiles from hexane soaks of (A) host fire ant worker; (B) *Orasema* sp. female adult from host ant colony; (C) *Orasema* sp. adult male from host ant colony. Alkaloids are designated by their side-chain length and whether or not they contain a double bond. The five species-specific hydrocarbons associated with *S. invicta* are defined by chromatogram section A.

1975; Nelson et al., 1980; Thompson et al., 1981) of the five major *S. invicta* hydrocarbon components identified as *n*-heptacosane, 13-methylheptacosane, 13,15-dimethylheptacosane, 3-methylheptacosane, and 3,9-dimethylheptacosane. Venom alkaloids leached from the poison sac of the host during the hexane soak are evident. Although it is known that *S. invicta* aerosols venom on its brood (Obin and Vander Meer, 1985), the amount of alkaloids present on individuals is too small to measure (< 1 ng) by GC; consequently, alkaloid peaks are absent or not detectable from the *Orasema* sp. soaks. Volatile components were not found when the starting GC oven temperature was 50°C (temperature program 1) and no additional peaks were found on maintaining the maximum oven temperature for a prolonged period of time (285°C for 22 min).

Gas chromatographic analysis of host colony *S. invicta* pupae and parasite wasp pupae were qualitatively identical (Figure 2A and B) as demonstrated by comparison of retention times and comparison of the mass spectrum of each peak. As in the case of the parasite adults, no additional highly volatile or nonvolatile peaks were found when different chromatographic conditions were used.

Several replicates of individual *Orasema* sp. pupae and adults from one colony were analyzed by GC. The percentage of each of the five major GC peaks were compared for host pupae and adult workers, and parasite pupae and adults of both sexes. The results are given in Table 1. Host and wasp pupae were indistinguishable for all five peaks. Host pupae and adult workers were significantly different in four of five hydrocarbon peaks. The male and female adult wasps were identical in all but one hydrocarbon peak; however, they were both significantly different from adult ants and wasp and ant pupae in three of five hydrocarbon peaks (not all the same peaks).

Adult *Orasema* sp., collected flying above *S. invicta* colonies, were analyzed by GC. A representative chromatogram is shown in Figure 3. Peaks characteristic of *S. invicta* hydrocarbons are evident (defined by retention times A on the chromatogram). However, in contrast to the chromatograms of the parasite taken directly from the host nest (Figure 1B and C; Figure 2A and B), the *Orasema* sp. adults captured outside host nests had complex GC peak patterns of both lower and higher retention times.

The results of the quantitative analysis of male and female adults from the host nest and males captured outside of the nest are shown in Table 2. The total amounts of GC detectable compounds from female and male *Orasema* sp. adults collected in the host nest are not statistically different; however, *Orasema* sp. males collected outside host nests had significantly more material than their within-nest counterpart (male or female). When only the hydrocarbon peaks characteristic of the host, *S. invicta*, are considered, males and females col-

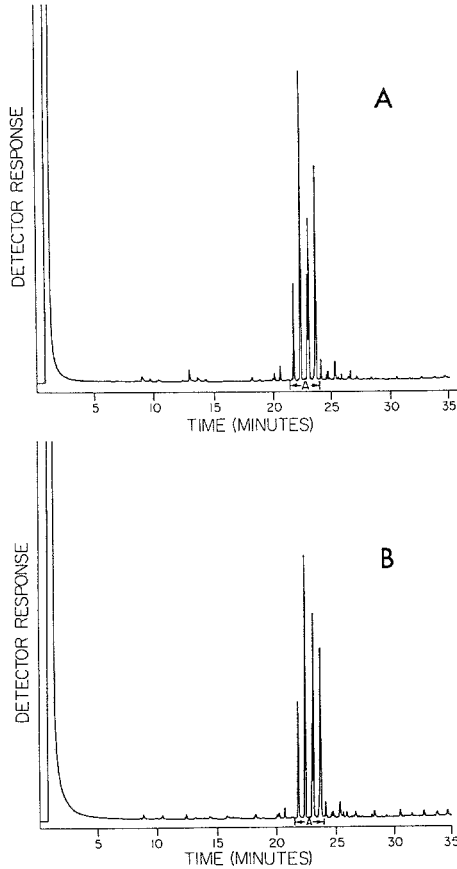


FIG. 2. Total gas chromatograph volatiles from hexane soaks of (A) host ant pupae and (B) *Orasema* sp. pupa from host colony. Chromatograph section A defines the species-specific hydrocarbons of the *S. invicta* host.

lected within the colony are indistinguishable; however, the *Orasema* sp. males collected outside the nest contain significantly less host hydrocarbon than the within-nest *Orasema* sp. The percentage of host-specific hydrocarbons to all GC components was 76.6% and 74.2% for the within-nest *Orasema* sp. females and males, respectively, and 14.5% for the *Orasema* sp. males collected outside the host colony.

The variance of the percentage of four new compounds (see Figure 3 and

TABLE 1. COMPARISON OF FIVE MAJOR GC COMPONENTS FOR PARASITE AND HOST PUPAE AND ADULTS^a

Sample (N)	Hydrocarbon Peak (in order of elution)				
	1	2	3	4	5
Host pupae (3)	10.4 ± 0.2 (A) ^b	34.4 ± 0.2 (A)	12.3 ± 0.2 (A)	16.5 ± 0.2 (A)	26.4 ± 0.2 (AB)
Wasp pupae (10)	8.9 ± 0.8 (A)	34.7 ± 0.7 (A)	11.7 ± 0.3 (A)	17.4 ± 0.6 (A)	27.3 ± 0.4 (AB)
Host adult (3)	16.4 ± 0.8 (B)	31.1 ± 0.3 (B)	9.9 ± 0.1 (B)	19.0 ± 0.4 (A)	23.6 ± 0.9 (C)
Wasp female (7)	9.2 ± 0.3 (A)	29.9 ± 0.6 (B)	10.9 ± 0.2 (B)	22.4 ± 0.5 (B)	27.6 ± 0.3 (A)
Wasp male (5)	10.6 ± 0.3 (A)	29.3 ± 0.7 (B)	10.2 ± 0.3 (B)	23.8 ± 1.0 (B)	26.1 ± 0.5 (B)

^a Mean and standard error of the percent of each of the five hydrocarbon peaks.

^b Means with different letters are significantly different ($P < 0.05$), Newman-Kuels test. Comparisons are made only within each column.

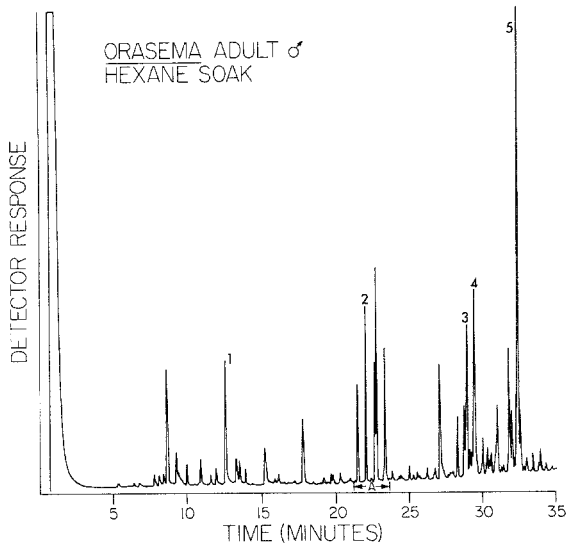


FIG. 3. Total gas chromatograph volatiles from a hexane soak of an *Orasema* sp. adult collected outside the host colony. Chromatogram section A defines the species-specific hydrocarbons of the *S. invicta* host. Peaks 1–5 were used to compare the variance of host- and nonhost-derived components.

Table 3) and one host-specific compound were compared. The relative proportions of the four new components were not significantly different from each other; however, in all cases the variance of the host-specific compound was greater than the four new components and in two cases significantly so.

TABLE 2. QUANTITATIVE COMPARISON OF GC COMPONENTS DERIVED FROM *Orasema* SP. ADULTS COLLECTED FROM WITHIN AND OUTSIDE HOST NEST

<i>Orasema</i> sp. type	All components (ng) ^a	Host-specific components (ng) ^a
Female from nest	602.3 ± 122.5 a ^b	461.4 ± 101.6 a ^b
Male from nest	450.3 ± 62.1 a	334.2 ± 42.8 a
Female outside nest	1340.9 ± 198.1 b	194.4 ± 122.1 b

^a Mean ± SD (*N* = 3); see Figures 1 and 3 for GC profiles.

^b Column results with a different letter are significantly different. Newman-Keul's test (*P* < 0.05).

TABLE 3. COMPARISON OF PERCENT COMPOSITION OF FOUR NONHOST COMPONENTS AND ONE HOST-SPECIFIC COMPONENT FROM *Orasema* SP. CAUGHT OUTSIDE HOST NEST

Component ^a	$X (\%) \pm SD (N = 9)^b$	Variance
1	8.83 \pm 2.33	4.82
2	8.77 \pm 5.32	25.11
3	15.60 \pm 1.90	3.18
4	24.46 \pm 3.50	10.90
5	42.44 \pm 3.06	8.31

^a See Figure 3.

^b Comparison of variance showed no significant difference between components 1, 3, 4, and 5; however, two (host-specific compound) was significantly different from components 1 and 3 ($P < 0.05$).

DISCUSSION

A great diversity of myrmecophiles have evolved a wide range of mechanisms for survival among their ant hosts (Wilson, 1971). The release of appeasement substances used to mitigate ant attacks and to gain entrance to a host colony is common (Hölldobler, 1971, 1972). Repellent and other defensive secretions are also employed (Brand et al., 1973; Gnanasunderam et al., 1981). One staphylinid beetle even mimics the alarm pheromone of its hosts, which aids in eluding worker ant attacks (Kistner and Blum, 1971). All of the above integration methods involve the release of myrmecophile-produced exocrine gland products. There are no reports of defensive or appeasement secretions for *Orasema* spp. or Eucharitidae in general. All information on the biology of the parasite states that they are not aggressively treated by the ants (Arye, 1962; Clausen, 1940; Wojcik, 1989). Past studies and observations of *Orasema* spp. parasites in the laboratory indicate that there are no defensive or appeasement secretions involved in their acceptance into fire ant colonies. We assume that initial entry into the colony by planidia is facilitated by their extremely small size (0.12–0.20 \times 0.04–0.07 mm; Heraty and Darling, 1984).

Morphological mimicry is also used by certain myrmecophiles (Wilson, 1971); however, to the human eye *Orasema* sp. larvae, pupae, and adults are easily distinguished from their host. Current evidence indicates that integration involves the nestmate-recognition mechanism of their ant host.

Colony odor is composed of heritable and environmental odors. Nestmate-recognition cues are a subset of colony odor and may be any combination of the heritable and environmental factors (Vander Meer, 1988). Colony odors of all types are continually being transferred to and from the cuticle of an individ-

ual ant via simple movement within the colony and social interactions with other workers, queen, and brood. This has been demonstrated with *S. invicta* (Sorensen et al., 1985) and other ant species (Errard, 1986). *S. invicta* and probably all *Solenopsis* spp. utilize a combination of environmentally and heritably derived nestmate-recognition cues (Obin, 1986).

The surface of an insect's cuticle is coated with lipids (Blomquist and Dillwith, 1985), which are ideal for the absorption of both environmental and heritable odors. Thus, each ant is enveloped in chemicals that identify it at species and colony levels (Vander Meer, 1983). The cuticular hydrocarbons of *S. invicta* are species specific and have been used as chemotaxonomic markers in several studies (Vander Meer et al., 1985; Vander Meer, 1986). The hydrocarbons represent a heritable component of colony odor; however, there is no direct evidence that they play a role in *S. invicta* nestmate recognition (Obin, 1986). Most important, however, is the fact that these easily analyzed (by gas chromatography) cuticular components can be used as markers for the movement of odors in a colony (Vander Meer, 1988). Vander Meer (unpublished) used the technique to demonstrate the dynamic nature of nestmate-recognition cues. This is further exemplified by the ability of the myrmecophilous scarab, *Martinezia duterteri*, to integrate into the nests of several *Solenopsis* sp. (Vander Meer and Wojcik, 1982). The mode of initial entrance into a *Solenopsis* colony is unknown; however, the beetle uses its armored exoskeleton and thanatosis to survive worker attacks long enough to acquire the host's colony odor. The acquisition of colony odor was monitored by GC analysis of ant cuticular hydrocarbons transferred to the cuticle of the beetle.

Based on our knowledge of the life history of *Orasema* sp., and their cuticular chemistry, we can postulate two possible integration mechanisms, one of which is analogous to that of *M. duterteri*. In this case the tiny planidia are transported undetected to the host colony. By some mechanism (probably through worker transfer during trophallaxis) they find their way to a *Solenopsis* brood chamber where they proceed to parasitize fire ant brood and develop into adults. *Orasema* sp. brood are treated by host workers as ant brood and, through social interaction with host workers and contact with host brood, they acquire host colony odors, ergo nestmate-recognition cues. This scenario is supported by comparative GC analysis of *Orasema* sp. life stages with those of their host (Figures 1 and 2). The GC profiles of the parasite collected within host colonies are remarkable in that they contain only components found in the host. This is in contrast to *M. duterteri*, which, in addition to the host components, had its own characteristic profile (Vander Meer and Wojcik, 1982).

Perhaps the *Orasema* sp. does not produce cuticular hydrocarbons in sufficient quantity to be GC detectable and is masked by the copious amount of hydrocarbons transferred from the host (hydrocarbons comprise 75% of the total cuticular lipid for *S. invicta*; Lok et al., 1975). Cuticular lipids are known to

be important in preventing desiccation in insects (Blomquist and Dillwith, 1985). This function can be carried out by hydrocarbons, wax esters, or combinations of these or other lipid classes. The *Orasema* sp. may rely on nonhydrocarbon lipids for prevention of desiccation, or they may require the acquisition of host lipids.

An alternative possibility involves biosynthesis by *Orasema* sp. of the exact hydrocarbon profile of the host, as reported for the termitophile *Trichosenius frosti* (Howard et al., 1980). Mediating against this hypothesis is the fact that environmental odors play an important role in *Solenopsis* spp. nestmate recognition (Obin, 1986; Obin and Vander Meer, 1988). This detracts from the adaptive advantages of biosynthesis by *Orasema* sp. of genetically derived colony odor components. One would predict that in ant species where genetically derived nestmate-recognition cues dominate, i.e., *Pseudomyrmex ferruginea* (Mintzer and Vinson, 1985), the probability of finding myrmecophiles that biosynthesized host recognition cues would be much greater.

In addition, results of the colony study (Table 1) demonstrate that GC profiles of host workers and pupae are distinguishable within a given colony. Although the profiles of parasite pupae are identical to those of host pupae, the adult parasite corresponds to a profile between that of the host pupae and adult. This can be readily explained by a preponderant transfer of brood cuticular components to other brood or objects among the brood (i.e., *Orasema* sp. brood). After eclosure of *Orasema* sp. adults, their contacts are predominantly with host workers.

The thanatotic behavior on the part of adult *Orasema* sp. in the host nest fits well with the hypothesis that nestmate-recognition cues, along with the lack of bidirectional agonistic behavior, explain the preferential treatment of ant brood by workers and the successful interspecific adoption of brood (Morel and Vander Meer, 1988). Similarly, when ant callow workers are transferred to a conspecific colony, or that of a closely related species, they respond by taking on a nonaggressive posture (Morel and Vander Meer, 1988; Jaisson, 1985).

The GC profile of adult *Orasema* sp. captured outside host colonies still contains host-specific hydrocarbons; however, they now also have additional complex patterns at both higher and lower molecular weights (Figure 3). Quantitative analysis of adult *Orasema* sp. (Table 2) demonstrates that the total amount of detectable compounds increases for parasites collected outside the nest by more than a factor of two and that the host-specific compounds decrease significantly after the parasite leaves the host nest. The loss of host-specific compounds on parasite adults caught outside the nest supports our contention that they are acquired and not biosynthesized by the parasite while it is living in the host nest. As in the case of the within colony parasite, the additional compounds found in parasites captured outside the nest could be acquired or biosynthesized.

If the new compounds are biosynthesized, the relative ratios of the new components to each other should be consistent, whereas the ratio of new components to host-specific components (assuming they are acquired) should be more variable. Alternatively, after leaving the ant colony, the adult wasp may acquire the new components from its new environment. Since *Orasema* spp. are not host-plant specific (Lloyd R. Davis, Jr., USDA-ARS, Gainesville, Florida, personal communication), the sources and therefore the compounds acquired from the environment would be expected to be variable. Chemical analysis of 10 individual *Orasema* sp. collected with a sweep net outside a fire ant colony showed remarkable consistency, both qualitatively and in percent composition (Figure 3, Table 3). Given the two possibilities outlined above, this implies that the new compounds found on *Orasema* sp. outside the host nest are probably biosynthesized by the parasite. In addition, the greater variance in percentage of the host-specific compounds on *Orasema* sp. collected outside the nest supports our contention that these compounds are acquired from the host and diminish when the parasite leaves the nest.

The data support a complex scenario for parasite cuticular chemistry. The immature parasites appear to acquire the recognition-masking colony odors of the host. In contrast, the GC profiles of adults found outside the mound suggest that the biosynthesis of parasite-specific cuticular compounds commences with adult eclosion.

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INHIBITION OF ATTRACTION TO AGGREGATION
PHEROMONE BY VERBENONE AND IPSENOL
Density Regulation Mechanisms in Bark Beetle
*Ips typographus*¹

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Abstract—The semiochemicals verbenone (Vn), ipsenol (Ie), and ipsdienol (Id), present in late phases of host colonization, have been implicated as qualitative “shut-off” signals regulating attack density. Combinations of the three chemicals were released in pipe traps together with the aggregation pheromone components 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol (cV) at different levels and in different ratios to MB + cV, and with two spacings of traps to test for possible effects on reducing catch at traps baited with aggregation pheromone. When they were released with the attractants Vn and Ie (alone or together) decreased the mean catch significantly at the higher release rates used (1 mg/day). Id alone or together with Vn at low release rates (0.1 mg/day), with the attractants, increased catch somewhat. A dose-response test of Vn, with the attractants held constant, showed a decline in catches, down to about <10% of the control, at ratios of Vn to cV between 1:1 and 150:1. A larger spacing (25 m) of traps gave a stronger response to change in doses of Vn and MB + cV than a smaller (6 m) spacing. The sex ratio was more skewed towards females when two or three inhibitors were present and at higher doses of Vn. It is suggested that Vn could be the most important density-regulating signal in the natural system, as release of Vn from galleries is larger and starts earlier than that of Id and Ie.

¹This study was made within the Swedish project “Odour signals for control of pest insects.”

Key Words—Aggregation pheromone, *Ips typographus*, Coleoptera, Scolytidae, density regulation, inhibitor, verbenone, ipsenol, ipsdienol.

INTRODUCTION

The eight-spined spruce bark beetle of Europe, *Ips typographus* (L.), regularly mass attacks standing, healthy host trees (Postner, 1974). In this respect, its population biology resembles the more aggressive Nearctic *Dendroctonus* species. The attack dynamics of the spruce bark beetle, i.e., the aggregation and density regulation process of initial attacks, mass attack, and then switching of attack focus to nearby trees, is only partly understood. The first part of the attack dynamics, where density is positively regulated by the attraction of both sexes to the aggregation pheromone released by the pioneering males, is empirically relatively well known (Bakke, 1976; Bakke et al., 1977; Bakke and Riege, 1982; Birgersson et al., 1984; Schlyter and Löfqvist, 1986; Schlyter et al., 1987a–c). A simple numerical model using these data (Schlyter and Anderbrant, 1989) described the first part of the process governed by the rapid release of 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol (cV) from the males, up to and including the mass attack, but after this period the model was inadequate (Anderbrant et al., 1988).

The latter part of the dynamics includes a decline in attraction and the switch of attack focus in order to avoid the well-established negative effects of larval competition at high densities on the number and quality of offspring (Thalenhurst, 1958; Ogibin, 1973; Botterweg, 1983; Anderbrant et al., 1985; Mills, 1986). The mechanisms governing the latter part are much less known, but two mechanisms have been proposed in *Ips typographus* (Schlyter et al., 1987c): (1) the quantitative hypothesis based on sex-specific response of males to large amounts of pheromone (relative inhibition; Byers, 1983) and the decrease of pheromone quantity after mating (Byers, 1981; Birgersson et al., 1984; Schlyter and Löfqvist, 1986), and (2) the qualitative hypothesis based on male production in later "attack phases" of ipsdienol (Id) and ipsenol (Ie) (Birgersson et al., 1984, 1988; Schlyter et al., 1987c) as inhibitors of the attraction to the pheromone. These might function as a "shut-off" signal for the aggregation. Ie is more or less inhibitory at the doses tested (Bakke, 1981; Schlyter et al., 1987c). In contrast, Id is inhibitory only at higher doses, but slightly attractive (Bakke, 1976; Schlyter et al., 1987c) or neutral (Dickens, 1981) at lower doses.

The numeric model of attack dynamics (Schlyter and Anderbrant, 1989) indicated that production of Id and Ie may start too late to influence the dynamics of density regulation (limitation) and switching at the rate these processes proceed in nature. It is likely that other signals are present in the process. One such additional signal may be the release of verbenone (Vn), which (at an

unspecified release rate) alone or together with Ie, has been shown to reduce the catch of *I. typographus* (Bakke, 1981). Verbenone also inhibits response (lower the catch at attractant baits) of North American species (*Dendroctonus frontalis*, Payne et al., 1978; *D. brevicomis*, Bedard et al., 1980; Tilden and Bedard, 1988; *D. ponderosae*, Ryker and Yandell, 1983; *D. adjunctus*, Livingston et al., 1983; *Ips paraconfusus*, Byers and Wood, 1980). The amounts of oxygenated monoterpenes, such as Vn, are found to increase in gallery wall phloem during late attack phases of *Ips typographus* and are probably produced by microorganisms, such as yeasts, brought to the tree by the beetle (Leufvén et al., 1984, 1988; Leufvén and Birgersson, 1987). The release of Vn from beetle-infested logs (Schlyter et al., 1987a) or from male attacks on standing trees (Birgersson and Bergström, 1989) is relatively high, often of the same magnitude as cV. Since Vn may affect behavior of the beetle, but is produced mainly by associated organisms (Leufvén et al., 1984) and only in trace amounts by the beetle (Birgersson et al., 1984), we use the term "semiochemical" for Vn together with Ie and Id. The two later compounds may also be termed pheromone components.

In order to test the relative importance of these three semiochemicals as "inhibitors" of attraction, we have released them, in racemic form, in all the seven possible combinations at two levels of release rates with the attractants MB + cV. We have also quantified the response to three decadic steps of Vn dose with MB + cV at two levels and spacing of traps in a factorial experiment.

METHODS AND MATERIAL

Compounds, Dispensers, Traps, and Study Area. The three proposed "inhibitors," which are all chiral molecules, were tested in racemic or close to racemic mixtures. For verbenone (Vn), which is probably derived from the chiral precursor α -pinene, which has a very high between-tree variation in enantiomeric composition (Lindström et al., 1989), both enantiomers of Vn could be expected to be equally active, as was also indicated by Bakke (1981). The Vn used was not far from racemic as it had a rotation (measured by polarimetry) of only $[\alpha]_D^{25} = +93.9^\circ$ ($c = 0.82\text{g}/100\text{ ml}$, 99.5% ethanol) (corresponding to 34.9% ee of *R* or 67.5 : 32.5% *R/S*). The two compounds derived from the achiral precursor myrcene in nature, ipsenol (Ie) and ipsdienol (Id), could be produced by the beetle in nonracemic form (Francke et al., 1980), but were synthetic and racemic.

The attractants 2-methyl-3-buten-2-ol (MB) and (4*S*)-*cis*-verbenol (cV) and their dispensers were identical to those earlier used by Schlyter et al. (1987a,c) (Table 1). Release rates were estimated in a mini wind tunnel in the laboratory as described earlier (Schlyter et al., 1987a).

TABLE 1. CHEMICALS, RELEASE RATES, AND DISPENSERS USED IN FIELD TEST OF INHIBITORY EFFECTS OF SEMIOCHEMICALS ON ATTRACTION TO AGGREGATION PHEROMONE IN *Ips typographus*, DENMARK, 1985

Compound	Purity (%)	Source	Release rate (mg/day)			Dispensers ^b
			Nominal	Measured ^a		
				Mean	95% C.L.	
Attractant aggregation pheromone components						
MB	97	Aldrich	5	5.8 ± 0.3		Closed #730 w. 50- μ l cap. 2-mm-diam., 3-ml vial
			50	57 ± 0.8		
cV	99	Borregaard	0.1	0.052 ± 0.001		Closed #730 w. 150- μ l cap. 9-mm-diam. hole, 2-ml vial
			1.0	1.03 ± 0.05		
Semiochemicals						
Vn	99	KTH, Stockholm	0.1	0.11 ± 0.02		Closed #730 Two open #730 Three open, 3-ml vials
			1.0	1.1 ± 0.03		
			10	7.7 ± 0.4		
Ie	99	Borregaard	0.1	0.1 ± 0.02		Closed #730 Open #730
			1.0	0.71 ± 0.09		
Id	95	Borregaard	0.1	0.1 ± 0.01		Two closed #730 Two open #730
			1.0	1.2 ± 0.1		

^aMeasured in a mini wind tunnel at 20°C and 0.7 m/sec. Rate calculated as the slope \pm its 95% confidence limits of the regression of weight loss vs. time.

^bDispensers were polyethylene vials that were either closed or closed but with hole drilled through the cap (x -mm-diam. hole) or with a capillary through the lid (w. x - μ l cap.) or open. Vials were from Kartell, Italy, with the #730 being a 1-ml vial.

Traps were of type N79, i.e. black drainpipe traps without exterior funnel (Regnander and Solbreck, 1981; Bakke et al., 1983). These traps require beetles to orient, land, and enter holes to be caught. Tests were done in three old clear-cut felling areas (S, L, and J), all with relatively low beetle populations in Esum Forest District, Grib Skov, North Zealand, Denmark, in May-June 1985. Randomization was "without replacement" (in the probability statistics sense) and followed a Latin-square design, i.e., with eight treatments and positions, a trap was assigned a random position after each replicate, but was tested exactly once in each position during eight replicates.

Qualitative Test of Verbenone, Ipsenol, and Ipsdienol Combinations. Vn, Ie, and Id were released in all possible combinations (singly or in binary or

trinary combinations) with the attractants, which with the control included eight treatments. Traps were placed at 6-m spacings in a clear-cut (site S), and their positions randomized after each replicate. A replicate, which lasted one day or longer, depending on flight activity, was completed when at least one trap was judged to have caught ≥ 50 beetles or when all traps had caught ≥ 100 beetles. For the first period (May 18–26, eight replicates), the “inhibitory” compounds were released at about 0.1 mg/day and for the second period (May 27–June 6, nine replicates) at about 1 mg/day (Table 1). In both cases, the control was a “low” level of attractant, MB + cV, at a release of 5 + 0.1 mg per day (Table 1).

Dose-Response Test of Verbenone. A release dose range of approximately 0.1, 1, and 10 mg/day of Vn (exact laboratory rates in Table 1) was combined with two levels of attractant, MB + cV at 50 + 1 mg/day and 5 + 0.1 mg/day, giving, with the two levels of attractants alone as controls, eight different baits. A 6-m spacing between traps was used for the two first replicates at each of the two sites (L and J) but was changed to a larger spacing (25 m) at site J, as the difference in catch between the two levels of attractant control baits was smaller than found in earlier studies (Schlyter et al., 1987c). Replications and randomizations were as described above, with the exception that the two first replicates at each site lasted for only half a day.

Statistics. The mean catch (\bar{x}) of a bait (i) per replicate (j) is given as the proportion (p_{ij}) of the total catch per replicate (p_j) in Figures 1 and 2 below. To further facilitate comparisons between the different experiments, the proportion of total catch caught by a bait, p_i , is given in percent of the catch (100%) of the “low” control bait (release rate level MB + cV, 5 + 0.1 mg/day). This “low” control has been used in several earlier experiments (Schlyter et al., 1987a–c; Byers et al., 1989) and has about 10% of the release of commercial mass-trapping dispensers (Ipslure by Borregaard/Hercon; Schlyter et al., 1987a).

Following Perry (1986) and Jones and Matloff (1986), we have used factorial MANOVA for analysis of the dose-response test. The variable used in ANOVA (Perry, 1986) was p_{ij} transformed by $\arcsin p^{0.5}$, which was the transformation that gave the most homogenous variances as judged by Cochran’s C test.

RESULTS

Qualitative Test of Effects of Verbenone, Ipsenol, and Ipsdienol Combinations. The higher release rate (1 mg/day) of the three compounds, together with a constant release of the attractants, resulted in a reduced total catch for all combinations of “inhibitors” (Figure 1B). Except for Id alone, the reduction was significant. The pattern of qualitative influence by individual compounds

and their combinations on catch was quite similar between the two release rates of semiochemicals (Figure 1).

However, when the three compounds were released at their low rate (0.1 mg/day) alone or in any combination with attractant, only small and not statistically significant negative (inhibitory) effects on catch were seen (Figure 1A).

In all combinations and at both levels, addition of Id seemed to increase catch (low release rate) or at least not significantly decrease catch (high rate).

With the low rate of inhibitor candidates, all the binary and trinary combinations decreased the proportion of males down to around 22% at 6 m trap spacing (Table 2A), while the total catch was little affected (Figure 1A).

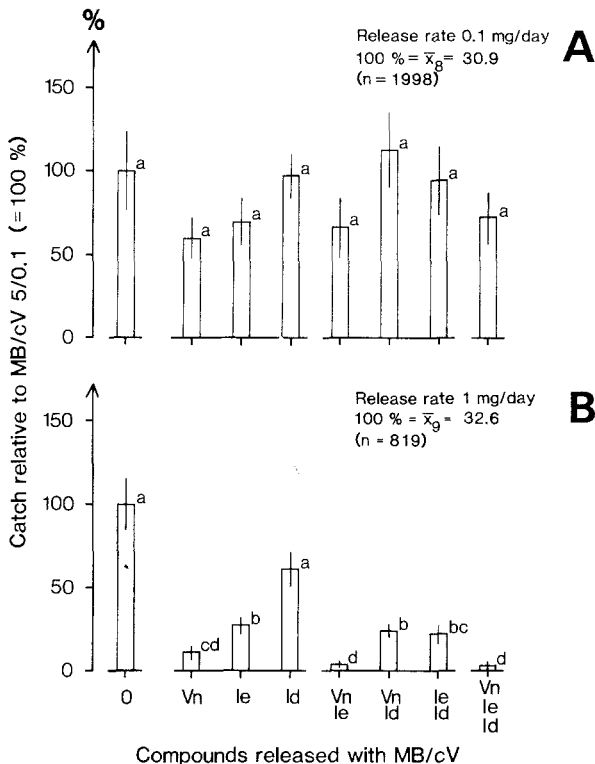


FIG. 1. Mean relative catches ($p \pm SE$) of *Ips typographus* in pipe traps at 6-m spacing with qualitative addition in all possible combinations of verbenone (Vn), ipsenol (Ie), and ipsdienol (Id) in separate dispensers to attractant pheromone (MB + cV at "low" release 5 + 0.1 mg/day). Catches expressed as percent of the catch of the control ("low" level of the attractants MB + cV). Bars with the same letter are not significantly different by ANOVA on arcsin $p^{0.5}$ followed by Duncan's multiple-range test. (A) Added semiochemical compounds (Vn, Id, Ie) released at approx. 0.1 mg/day, (B) semiochemicals released at approx. 1 mg/day (see Table 1 for exact laboratory estimated release rates). For sex ratios, see Table 2.

TABLE 2. RESPONSE OF SEXES OF *Ips typographyus* TO TESTS OF VERBENONE (Vn), IPSENL (Ie), AND IPSDIENOL (Id) WITH ATTRACTANT PHEROMONE IN GRIB SKOV, DENMARK, 1985

Treatment release rates (mg/day)						Response	
MB	cV	Vn	Ie	Id	Ratio (Vn + Ie + Id)/cV	% Males	
						Mean (N)	95% CI
A. Combinations of compounds added at 0.1 mg/day, 6-m spacing, site S, May 18-26 (8 replicates)							
Control							
5	0.1					35.4 (237)	29.6-41.7
One compound							
5	0.1	0.1	0	0	2.1	30.1 (191)	24.1-36.9
5	0.1	0	0.1	0	1.9	33.3 (195)	27.1-40.2
5	0.1	0	0	0.1	1.9	35.5 (248)	29.8-41.6
Two compounds							
5	0.1	0.1	0.1	0	4.0	22.5 (169)	16.8-29.4 ^a
5	0.1	0.1	0	0.1	4.0	21.5 (326)	17.4-26.2 ^a
5	0.1	0	0.1	0.1	3.9	22.1 (263)	17.5-27.4 ^a
Three compounds							
5	0.1	0.1	0.1	0.1	6.0	24.7 (186)	19.1-31.3
All baits						27.1 (1504)	25.0-29.5
B. Combinations of compounds added at 1 mg/day, 6-m spacing, site S, May 27-June 6 (9 replicates)							
Control							
5	0.1					34.8 (293)	29.6-40.4
One compound							
5	0.1	1	0	0	21	26.0 (34)	15.0-43.0
5	0.1	0	1	0	14	22.0 (85)	15.0-32.0
5	0.1	0	0	1	23	35.6 (222)	29.6-42.1
Two compounds							
5	0.1	1	1	0	35	(6.0) (16)	1.0-28.0 ^a
5	0.1	1	0	1	44	26.0 (89)	18.0-36.0
5	0.1	0	1	1	37	29.0 (69)	20.0-41.0
Three compounds							
5	0.1	1	1	1	58	(33.0) ^b (12)	14.0-61.0
All baits						31.3 (820)	28.3-34.6
C. Dose-response test of Vn, 25-m spacing, site J, May 17-June 4 (12 replicates)							
Control, low-level attractants							
5	0.1					45.9 (98)	36.4-55.6
Vn dose with low-level attractants							
5	0.1	0.1			2.1	34.0 (79)	25.0-45.0
5	0.1	1			21.0	(50) (16)	28.0-72.0
5	0.1	10			148.0	(0) (7)	0-66
Control, high-level attractants							
50	1					24.6 (378)	20.5-29.2
Vn dose with high-level attractants							
50	1	0.1			0.11	18.9 (275)	14.7-24.0 ^a
50	1	1			1.1	26.5 (215)	21.1-32.8 ^a
50	1	10			7.8	31.0 (52)	20.0-44.0

^aSignificantly different from the control (low level attractants) at $P < 5\%$.

^bValues for % males within parentheses are based on less than 20 individuals.

For the high rate of “inhibitors,” the proportion of males was very similar for both the control and addition of Id alone, while for the addition of Vn + Id, the male proportion was significantly lower (6% males, Table 2B). However, the absolute catches were low, making estimates of sex-ratio differences less reliable.

In contrast to the spruce bark beetle, its predator *Thanasimus formicarius* was mostly attracted only to the bait including Id + Id, which had 38% of the total 26 clerids caught.

Verbenone Dose-Response. Verbenone (Vn) decreased trap catch when released at 1 mg/day or more with both levels of attractants (Figure 2). At a 25-m spacing of traps, a significant inhibition began to occur at a ratio of Vn to cV of 0.1 : 1 (Figure 2B), corresponding to a ratio of Vn to Mb + cV as low as 0.002 : 1.

Of the factors (ANOVA) in the experiment, the level of attractant (MB + cV) had the largest main effect, followed by the dose of verbenone (Table 3). The factor spacing between traps had very little effect, as would be expected when relative catch, p_{ij} , per bait (i) per replicate (j), was the variable used in the analysis. The interaction of spacing with attractant level (Table 3) can be

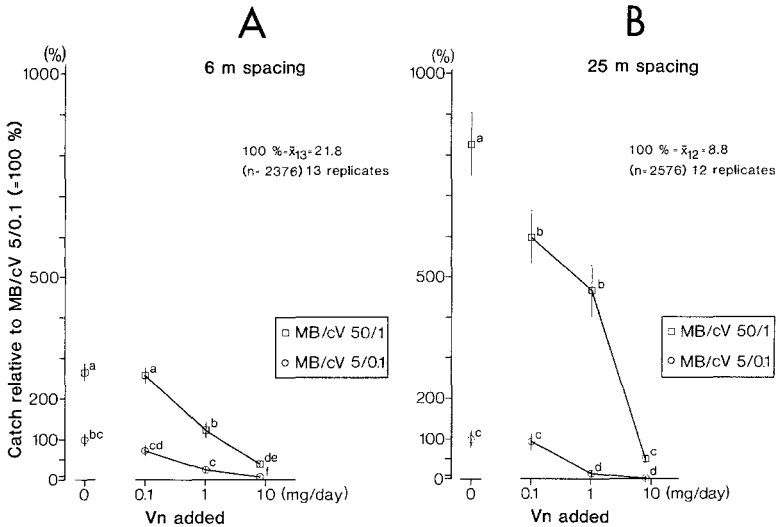


FIG. 2. Mean relative catches ($p \pm SE$) of *Ips typographus* in pipe traps with doses of verbenone (Vn) added to two levels of attractants (MB + cV) at (A) 6-m spacing, and (B) 25-m spacing of traps. Catches expressed as percent of the catch of the “low” control (MB + cV, 5 + 0.1 mg/day) (cf. Figure 1). Within each spacing of traps, values with the same letter are not significantly different at $P > 5\%$ by ANOVA on arcsin $p^{0.5}$ followed by Duncan’s multiple-range test.

TABLE 3. FACTORIAL MANOVA OF EFFECTS OF SPACING OF TRAPS, LEVEL OF ATTRACTANT (MB + cV), AND DOSE OF VERBENONE (0–10 mg/day) ON TRAP CATCHES OF *Ips typographus*, GRIB SKOV, DENMARK, 1985^a

Factor	df	Mean square	F	Significance of F (%)
Within cells	180	0.1		
Constant	1	18.1	1923.6	<0.1 ***
Main effects				
SPACE (Distance between traps) ^b	1	0.04	4.2	4.2 *
ATTLEVEL (Level of attractant) ^c	1	4.1	437.8	<0.1 ***
VNDOSE (Dose of verbenone) ^d	3	1.1	119.1	<0.1 ***
Interactions				
SPACE × ATTLEVEL	1	0.2	18.6	<0.1 ***
SPACE × VNDOSE	3	0.01	1.1	33.6 NS
ATTLEVEL × VNDOSE	3	0.1	14.0	<0.1 ***
SPACE × ATTLEVEL × VNDOSE	3	0.04	4.3	0.6 **

^aCatch was transformed by $\arcsin p^{0.5}$ before analysis. Test of homogeneity of variances yielded Cochran's $C(11, 16) = 0.13$, $P = 23\%$ (approx.) after transformation.

^bTwo levels: 6 and 25 m spacing of traps. The factor SPACE was confounded with possible effects of location, as SPACE was not balanced between the two locations: there were two runs (replicates of each site) of 6 m at both sites before the change to 25 m spacing at one site. The total number of replicates was 2 + 11 for 6 m spacing and 0 + 12 for 25 m spacing. The results of the two first replicates at each of the two sites were very similar in absolute and relative catches ($P \gg 10\%$ in ANOVA), and the age, size, and proximity to naturally attacked trees (>500 m) was also similar for the two sites. Thus, it was judged appropriate to do a simultaneous statistical comparison and to include the factor SPACE in the ANOVA.

^cTwo levels: MB + cV 50 + 1 or 5 + 0.1 mg/day (see Table 1).

^dFour levels: 0, 0.1, 1, and 10 mg/day (see Table 1).

clearly seen when comparing Figure 2A and B, as the difference in catch levels between attractant levels is much larger in Figure 2B (25-m spacing). There was also a quite significant covariance between attractant level and verbenone dose (Table 3), graphically detectable as the different slopes for high and low attractant levels with increasing dose of Vn (Figure 2A and B) (cf. Tilden and Bedard, 1988). Also the third-order interaction was significant (Table 3), which can be seen graphically by the difference in slope between the attractant levels, which was not the same for the two trap spacings (Figure 2A and B). A direct test of the ratio between MB + cV and Vn as a factor was not possible as the ratio did not vary independently of Vn dose and attractant level.

The sex-ratio differences were large at the 6-m spacing of traps. At the low level of attractant (MB + cV, 5 + 0.1), the sex ratio decreased from 1:0.9 (male to female) at the control to 1:3 (24–31% males) at the higher Vn doses.

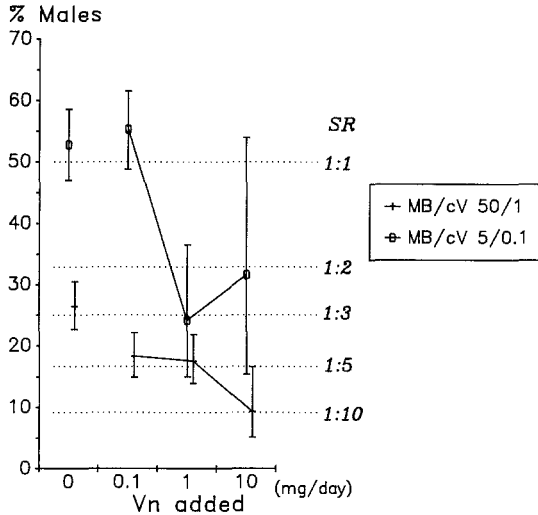


FIG. 3. Mean proportion of males ($p \pm 95\%$ C.I.) of *Ips typographus* in pipe traps with doses of verbenone (Vn) added to two levels of attractants (MB + cV) at the 6-m spacing. Values shown are retransformed from arcsin $p^{0.5}$, hence the asymmetric CI.

For the high level of attractant (MB + cV, 50 + 1), a threefold decrease in sex ratio, from 1:3.3 in the control to 1:10 (9% males) at the highest Vn dose, took place (Figure 3). At the 25-m spacing of traps, there was no effect on sex ratio by Vn dose alone (Table 2C).

DISCUSSION

The present study shows the inhibitory effects of ipsenol and verbenone on the attraction of *Ips typographus*. The effect is especially strong on the number of males, as both the total number of beetles and the proportion of males are reduced. The qualitative effects of Ie and Vn acting alone or in the pair Ie + Id were not unexpected as they agree with the findings of Bakke (1981) and Schlyter et al. (1987c). In the qualitative test Vn showed a negative effect on attraction, at both release rates (except in the binary combination with Id at the low rate). This agrees also with the effect of the binary combination of Ie + Vn with the attractants tested by Bakke (1981). Similar to previous reports (Dickens, 1981; Schlyter et al., 1987a, b), addition of Id to MB + cV had no significant effect on trap catches compared to MB + cV alone. Our current results do not show the effect shown in previous studies in which Id in combination with MB + cV was attractive at some dosages (0.005–0.04 mg/day,

Schlyter et al., 1987c; unspecified but relatively low doses, Bakke 1976; Bakke et al., 1977) but inhibitory at a high dose (0.58 mg/day, Schlyter et al., 1987c). Similar to the qualitative test, an increased dose of Vn (with both levels of attractant) decreased the trap catch linearly down to a level of 10% or less of the control (cf. Tilden and Bedard, 1988). The mechanism of this inhibitory effect of Vn is probably an inhibition of long-distance orientation, as the quantitative effect of verbenone dose was clearer at the 25-m than at 6-m spacing between traps (cf. Tilden and Bedard, 1988).

These experimental results must now be put into the framework of both how the semiochemicals (quantity and quality) are released from gallery systems in different attack phases (Birgersson et al., 1984; Leufvén and Birgersson, 1987; Birgersson and Bergström, 1989) and how they may affect the overall attack dynamics (Schlyter and Anderbrant, 1989; Anderbrant et al., 1988). The three compounds (Vn, Ie, Id) tested here were racemates or close to racemic, while their natural quality may be either as pure enantiomers or as mixtures with enantiomeric excess. For Vn, the racemate used is probably not a problem, as Vn chirality may depend on the pinan-skeleton of the α -pinene from which it is derived, and the chirality of α -pinene is known to vary widely between individual trees of Norway spruce (Lindström et al., 1989). Also, the activity of *R*- and *S*-enriched Vn tested by Bakke (1981) was equally high in reducing trap catch at the dose tested. For Id, however, it is possible that effects could have been distorted by the use of the racemate, because (1) the chirality of Id is known to be important in the behavior of *Ips* beetles (Birch et al., 1977, 1980; Wood, 1982), (2) Id produced by *I. typographus* has been reported as not racemic (Francke et al., 1980), and (3) it is known that a compound attractive in enantiomerically pure form may, as a racemate, show inhibitory effects at high release rates but attractive effects at low rates (Vité et al., 1985). However, we decided to test Id in racemic form as the compound was not available to us as pure enantiomers. In contrast, Ie seems to lack attractive effects at all rates tested both here and in a previous study (Schlyter et al., 1987c), and it would seem less likely that attractive properties of Ie were concealed by the racemic composition.

Estimates of the quantity of Ie, Id, and Vn released from logs (Schlyter et al., 1987a) and from galleries in attacked trees (Birgersson and Bergström, 1989) are now becoming available. These studies show that, although Ie and Id are undoubtedly present in beetle hindguts of males in late attack phases (Bakke, 1976; Birgersson et al., 1984, 1988), they are produced in such small quantities that they cannot be detected in the samples from airborne collections (Birgersson and Bergström, 1989). In contrast, Vn is readily detected and released in quantities similar to or larger than *cis*-verbenol from beetle infested logs and from galleries in trees (Schlyter et al., 1987a; Birgersson and Bergström, 1989).

The production mechanisms for the relatively large amounts of Vn, which

is found only in trace quantities in beetle hindguts (Birgersson et al., 1984, 1988), are not clear. Both autoxidation from α -pinene (Borden et al., 1986) and microbial mediated oxygenation of verbenols (Leufvén et al., 1984) have been shown. Irrespective of the relative and absolute release of the three semiochemicals (Id, Ie, and Vn), they could in theory all contribute to an odor message of an "old patch," as they are all produced in late attack phases by the beetle (Id, Ie) or by a sequence of oxidations of α -pinene in the defensive resin (Vn). At present, it seems that intraspecific attack density regulation by a qualitative change in the odor signal from a patch (such as a tree or a part of a tree) in *I. typographus* would be mostly dependent on Vn. The response of *I. typographus* to the beetle produced compounds (Id, Ie) may be more relevant in interspecific communication and competition, as Id and Ie are produced in larger quantities both by *Picea*-inhabiting sympatric *Ips* species such as *I. duplicatus* (Bakke, 1975) and *I. amitinus* (Francke et al., 1980) and by other sympatric species: *I. sexdentatus* (Vité et al., 1974), *I. acuminatus* (Bakke, 1978), *I. cembrae* (Stoakley et al., 1978), and *I. erosus* (Giesen et al., 1984). The attraction of the predatory *Thanasimus* beetles to baits with Ie and/or Id has been shown earlier (Bakke and Kvamme, 1981; Schlyter et al., 1987c).

An integration of the inhibitory effects of Vn and Ie into a quantitative model of attack dynamics (cf. Schlyter and Anderbrant, 1989) and into practical uses for forest protection would require more knowledge. Gathering of such data for time-dependent release (Birgersson and Bergström, 1988) and quantitative dose-response on attacks on logs (Schlyter et al., 1988) is now under way.

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COMPARATIVE DETERRENCY OF TWO TERPENOIDS TO TWO GENERA OF ATTINE ANTS

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Abstract—Caryophyllene and caryophyllene epoxide are two terpenoids found in neotropical plants and known to be deterrent to leafcutter ants. To estimate the variation in deterrent activity of these compounds toward the genera *Atta* and *Acromyrmex*, behavioral bioassays were conducted over a range of concentrations. The responses of four captive colonies of *Atta cephalotes* and two captive colonies of *Acromyrmex octospinosus*, all from a single locality in Costa Rica, were studied. Although specific patterns of deterrency differed in the two genera, in both cases caryophyllene epoxide concentrations of 0.70 mg/g or greater, and caryophyllene concentrations of 7.0 mg/g or greater, significantly deterred the harvest of potential substrates. Individual colonies within each genus did not differ significantly in their responses to caryophyllene epoxide. The mean response of the two genera to caryophyllene epoxide differed significantly, but responses to caryophyllene did not. *Acromyrmex octospinosus* was more sensitive to low concentrations and less sensitive to high concentrations of caryophyllene epoxide than was *Atta cephalotes*.

Key Words—*Atta cephalotes*, *Acromyrmex octospinosus*, leafcutting ants, Hymenoptera, Formicidae, terpenoids, deterrents, dosage-response studies, bioassays.

INTRODUCTION

Leafcutting ants of the genera *Atta* and *Acromyrmex* (Formicidae: Attini) are common generalist herbivores in neotropical forests. These ants harvest leaves from a variety of woody plant species in natural communities (Cherrett, 1968; Rockwood, 1976) and are significant pests throughout the Neotropics (Cherrett, 1986a). Current methods of chemical control are costly and may have undesirable effects on nontarget species (Cherrett, 1986a, b; Vilela, 1986). The search for improved methods of control has led us to study natural mechanisms of resistance in native plants.

Recent studies of the leafcutting ant *Atta cephalotes* in tropical deciduous forest of Costa Rica suggest that plant secondary chemistry is an important influence on the preferences of leafcutters for plants (Hubbell et al., 1984; Howard, 1987, 1988). These studies suggest that nonpolar compounds such as terpenoids strongly influence ant behavior. A number of hydrophobic natural products deter or repel *A. cephalotes* in laboratory bioassays (Wiemer, 1985), and at least some are toxic to the ants and/or to their fungal mutualist (Howard et al., 1988).

The bioassays done to guide the isolation of leafcutter ant deterrents were conducted using a single laboratory colony (*A. cephalotes* or *A. octospinosus*) for each plant studied. Such single-colony studies offer precise estimates of comparative chemical effects. All ants within these single-queen colonies are at least half sibs, and possibly full sibs, descendant from the same mother, and thus are genetically similar. However, there are valid reasons for studying colony-to-colony variation. Bioassay results from a single colony may not be representative of the species as a whole if genetic variation in ants or fungal strains produces significant colony-to-colony differences in response to chemicals. Estimates of colony-to-colony variation within and among habitats may offer insights into the structure of leafcutting ant populations. Finally, comparative dosage-response studies on leafcutter species and genera are important in determining the potential of natural products for chemical control of this economically important group of insects.

Here, we report a comparison of the deterrent activity of two terpenoids to colonies of the leafcutting ants *Atta cephalotes* L. and *Acromyrmex octospinosus* Reich. These species are widely sympatric in Central America, and the two genera are thought to forage on similar resources in their natural forest communities (Weber, 1972). Behavioral bioassays were used to measure the response of ant colonies to several concentrations of the two compounds. Our studies address the following questions: (1) Do colonies of a given species react similarly to these pure compounds? (2) Do different leafcutting ant genera react similarly? (3) What is the minimum concentration of these chemicals required to significantly deter substrate harvest by ants of these genera?

METHODS AND MATERIALS

Studies were conducted on four colonies of *Atta cephalotes* and two colonies of *Acromyrmex octospinosus*. All colonies were collected in July 1982 from a tropical deciduous forest in Santa Rosa National Park, Guanacaste Province, Costa Rica, and contained a single queen. Colonies of *Atta cephalotes* all possessed a single small entrance at the time of collection, indicating that they were approximately one year of age (Weber, 1972). Colonies of *Acromyrmex octospinosus* were of a similar size at the time of collection but are in general smaller than those of *Atta* and may have been older than one year (Weber, 1972). All colonies were maintained in Plexiglas boxes in an insectarium at the Department of Biology, University of Iowa. The room was maintained at 23°C and 40–60% relative humidity, on a 12:12 light–dark cycle. Colonies were fed fresh lilac leaves in season and frozen leaves during winter months. The colonies were maintained under these conditions for approximately one year prior to the start of bioassays. All experiments were conducted between July and October while colonies were being fed fresh, mature lilac leaves.

The two compounds selected for testing, caryophyllene and caryophyllene epoxide, are common secondary constituents of tropical plants (Arrhenius and Langenheim, 1983; Hubbell et al., 1983; Wiemer, 1985). Both compounds deter *Atta cephalotes* in laboratory bioassays, and are toxic to both ants and fungus (Howard et al., 1988). We obtained authentic samples from a commercial supplier (Aldrich) and tested five different concentrations of each compound on each colony. Although structurally similar, caryophyllene is 10–20 times less deterrent to leafcutting ants than caryophyllene epoxide (Hubbell et al., 1983). Accordingly, we tested caryophyllene epoxide at concentrations 10-fold lower than caryophyllene.

Standard behavioral bioassays were used to measure the deterrency of each concentration of the two chemicals on each colony (Hubbell and Wiemer, 1983). Prior to use in a bioassay, rye flakes were oven dried for 24 hr at 50°C and stored in a desiccator until needed. These rye flakes (0.6 g) were soaked for 1 min in a solution of the test chemical in methylene chloride (1.2 ml total volume), removed, air dried at room temperature, and presented to the colony on a computer-generated random grid. Rye flakes soaked in methylene chloride and dried in a similar fashion served as a control. Each bioassay included 60 test flakes and 60 control flakes. Ants were allowed to forage over the grid until 30 control flakes had been harvested; at this time the number of test flakes harvested was determined. The significance of differences between numbers of control and test flakes harvested ($C - T$) was determined using a modified binomial test (Hubbell and Wiemer, 1983).

We estimated the amount of test chemical actually deposited on rye flakes by measuring the amount of caryophyllene and caryophyllene epoxide remain-

ing in test solutions. After soaking the rye flakes, the remaining solution was returned to its original vial. The porcelain crucible used for soaking the flakes was rinsed with additional solvent and the rinse combined with the remaining solution. This was filtered to remove any particulate matter, and then dried and weighed (± 0.1 mg). The maximum amount deposited on test flakes was estimated by subtraction. In each case, analysis of the residual test solution by gas chromatography (OV-101, 50–270°C) revealed only a single peak corresponding to the test chemical, and quantification by GC agreed with the gravimetric analyses. This verified that methylene chloride did not extract significant amounts of material from the rye flakes.

Three replicate bioassays were carried out on each colony at each of the five concentrations of caryophyllene epoxide, totaling 90 bioassays. To avoid biases in response due to previous experience, the order of presentation was randomized for each colony and several days were allowed to elapse between consecutive tests on a single colony. A similar procedure was planned for caryophyllene. Unfortunately, an infestation of mites required suspension of experiments after completion of the first set of experiments (30 bioassays). Replicate assays were not possible because two of the *Atta* colonies died when mite infestations overwhelmed their fungus, bringing experiments to an end.

Data analyses were carried out using the SYSTAT (version 3.0) computer package for microcomputers. Data from the caryophyllene epoxide experiments were used to analyze the variation in response among colonies of each species, because these experiments were replicated at the colony level. An analysis of covariance was used because the concentrations tested were arbitrarily selected from a continuous range of possible concentrations. Concentration was treated as a continuous variable and each colony as a discrete class. Separate analyses were performed for *Atta cephalotes* and *Acromyrmex octospinosus*. The data were tested for homogeneity of variances among colonies and chemical concentrations and were found to require no transformation. Residuals from the analysis were examined for normality and influence.

The data from both caryophyllene epoxide and caryophyllene experiments were used to analyze differences in response between *Atta* and *Acromyrmex*. The response of each colony to each concentration of caryophyllene and the mean of the three replicates for each colony at each concentration of caryophyllene epoxide were treated as single observations in this analysis. Separate analyses of covariance were run for caryophyllene epoxide and caryophyllene experiments, treating chemical concentrations as continuous variables and treating ant species as discrete classes. Homogeneity of variance and residual analyses were conducted as previously described. A square-root transform was used to obtain homogenous variances in the caryophyllene analysis.

RESULTS

Slightly more than one third of the test chemical present in solution was deposited on rye flakes ($34.77 \pm 5.02\%$). Percent deposition of caryophyllene and caryophyllene epoxide on rye flakes did not differ ($T = 0.904$, $P > 0.2$, t test) and did not vary significantly with concentration ($r^2 = 0.043$, $P > 0.4$, simple regression). Accordingly, we applied a constant correction factor of 35% to all concentrations of caryophyllene and caryophyllene epoxide. We estimate that effective concentrations encountered by ants during bioassays were 0.07, 0.21, 0.70, 1.40, and 2.10 mg of caryophyllene epoxide per gram of rye flakes, and 0.70, 2.10, 7.00, 14.0, and 21.0 mg of caryophyllene per gram of rye flakes.

Under the conditions of our bioassay, the harvest of 16 or fewer test flakes was significantly different from control flake harvest ($P < 0.05$). Both *Atta cephalotes* and *Acromyrmex octospinosus* colonies harvested fewer test than control rye flakes at even the lowest concentrations of caryophyllene epoxide tested, although these differences were generally not significant (Figure 1). Mean test flake harvest by *Atta* colonies fell below 16 at caryophyllene epoxide concentrations of 0.70 mg/g or above. *Acromyrmex* colonies harvested 16 or fewer test flakes at all concentrations of 0.21 mg/g or above (Figure 1). A comparison of the overall responses of *Atta* and *Acromyrmex* to caryophyllene and caryophyllene epoxide showed that, as expected, caryophyllene epoxide produced responses similar to those produced by 10-fold higher concentrations of caryophyllene. Mean test flake harvest fell below 16 in both species when colonies were presented with caryophyllene concentrations equal to or greater than 7.0 mg/g (Figure 2).

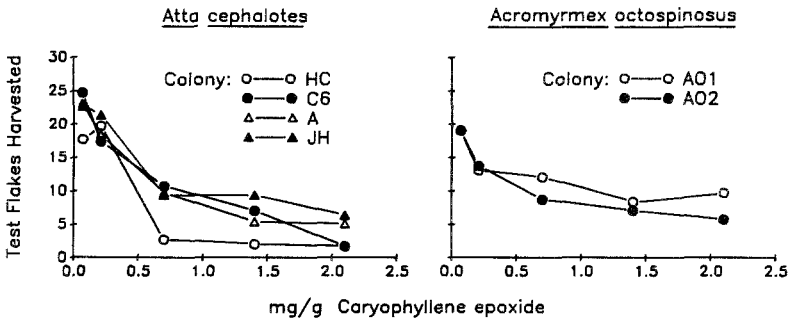


FIG. 1. Response of individual colonies of *Atta cephalotes* and *Acromyrmex octospinosus* to caryophyllene epoxide.

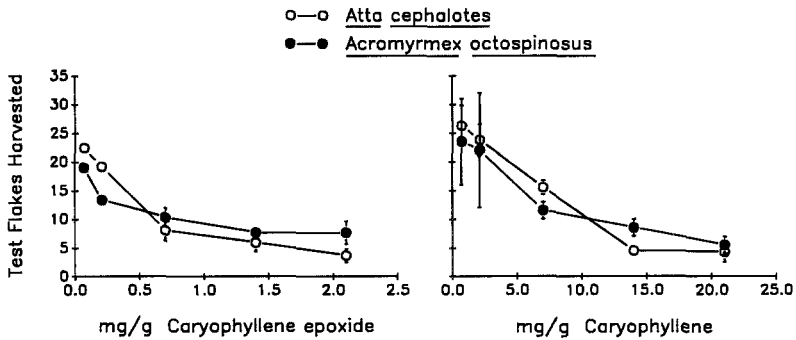


FIG. 2. Overall response of *Atta cephalotes* and *Acromyrmex octospinosus* to caryophyllene epoxide and caryophyllene. Bars are ± 1 standard error.

Analysis of covariance indicated that variation in deterrent activity of caryophyllene epoxide among colonies was not statistically significant in either species (Table 1) and that the slope of the relationship between test flake harvest and concentration was similar in all colonies of a species (colony \times concentration effect, Table 1). The effect of caryophyllene epoxide concentration was highly significant in both analyses, explaining 68.1% of the variation in test flake harvest by *Atta cephalotes* and 53.8% of the variation for *Acromyrmex octospinosus* (Table 1).

TABLE 1. ANALYSIS OF COLONY-TO-COLONY VARIATION IN RESPONSE OF *Atta cephalotes* AND *Acromyrmex octospinosus* TO CARYOPHYLLENE EPOXIDE AT FIVE CONCENTRATIONS

Source	Sum of squares	df	Mean square	F ratio	P
<i>Atta cephalotes</i>					
Colony	43.099	3	14.366	0.560	0.644
Concentration	2990.008	1	2990.008	116.526	<0.001
Colony \times Conc.	15.000	1	6.964	0.271	0.846
Error	1334.300	52	25.660		
Total	4388.299				
<i>Acromyrmex octospinosus</i>					
Colony	2.673	1	2.673	0.176	0.678
Concentration	481.667	1	481.667	31.678	<0.001
Colony \times Conc.	15.000	1	15.000	15.000	0.330
Error	395.333	26	15.205		
Total	894.673				

TABLE 2. ANALYSIS OF OVERALL RESPONSES OF *Atta cephalotes* AND *Acromyrmex octospinosus* TO CAROPHYLLENE EPOXIDE AND CARYOPHYLLENE

Source	Sum of squares	df	Mean square	F ratio	P
Caryophyllene epoxide					
Ant Species	58.970	1	58.970	6.386	<0.025
Concentration	833.820	1	833.820	90.298	<0.001
Species × Conc.	67.021	1	67.021	7.258	<0.025
Error	240.087	26	9.234		
Total	1199.898				
Caryophyllene ^a					
Ant Species	0.783	1	0.783	1.416	0.245
Concentration	34.063	1	34.063	61.569	<0.001
Species × Conc.	1.065	1	1.065	1.925	0.177
Error	14.384	26	0.553		
Total	50.295				

^aSquare root transformed data.

Analysis of overall responses of *Atta* and *Acromyrmex* indicated that the two genera differed significantly in their responses to caryophyllene epoxide (Table 2). Although higher concentrations were always more deterrent than low concentrations, the slope of the relationship between test flake harvest and concentration differed significantly between genera (species × concentration effect, Table 2). *A. octospinosus* was more sensitive to low concentrations and less sensitive to high concentrations of caryophyllene epoxide than *A. cephalotes* (Figure 2). Although the same qualitative patterns were observed in caryophyllene bioassays (Figure 2), no statistically significant differences were found in responses of the two species to caryophyllene (Table 2).

DISCUSSION

The responses of insects to chemical stimuli may be affected by genetic differences and by prior experience. Although the colonies tested in this study may have encountered different plants and secondary chemical defenses prior to collection, individual workers live only four to six months (Weber, 1972). Because colonies were maintained under standardized conditions in our laboratory for one year prior to the study, all workers in all colonies had identical recent histories of exposure to plants and chemicals. Possible seasonal variation in colony responses was minimized by maintaining colonies under constant

environmental conditions and by feeding all colonies fresh, mature lilac leaves during the months of these experiments. Any differences in response observed in this study should be due primarily to genetic differences among colonies or between species.

We found no statistically significant variation in the responses of colonies to given concentrations of caryophyllene epoxide in either ant species. The relationship between concentration and test flake harvest was also similar for all colonies within a species. This is consistent with evidence from field experiments suggesting that ant colonies within a single habitat or locality respond similarly to plant characteristics (Howard, 1987). However, the colonies tested in this experiment may have all originated from a single nuptial flight of a few *Atta cephalotes* or *Acromyrmex octospinosus* colonies and may represent only a fraction of the total genetic diversity present within populations at Santa Rosa National Park. Further experimentation on colonies collected from this and other sites will be required to establish the extent to which patterns of deterrent activity may be generalized within and among attine populations and species.

Atta cephalotes and *Acromyrmex octospinosus* differed significantly in their responses to caryophyllene epoxide but did not differ in response to caryophyllene. This may be due in part to the higher variance inherent in the caryophyllene measurements (Figure 2). These measurements are based on a single observation per colony, while the caryophyllene epoxide measurements are the mean of three replicates. *Acromyrmex octospinosus* was more sensitive to low concentrations and less sensitive to high concentrations of both test chemicals (Figure 2), and the similarity of these results suggests that the two genera may, in fact, differ for both chemicals tested.

The biological significance of differences in the response of the two genera is not immediately clear from the whole animal bioassays reported in this study. Whatever the physiological or biochemical mechanism involved, it is reasonable to hypothesize that differences in perception may be related to differences in ecological relationships between ants and terpenoid-containing plants, and it may be that the ecological roles of *Atta* and *Acromyrmex* are less similar than is currently supposed. Few studies of diet selection and foraging in forest-dwelling *Acromyrmex* species have been carried out compared to *Atta* or grass-cutting *Acromyrmex* from the savannahs of South America. Comparative studies of sympatric forest-dwelling *Atta* and *Acromyrmex* would be of particular interest in determining if the subtle differences seen in this study reflect substantial differences in life-style.

Although specific patterns of deterrence differed in the two genera, effective concentrations of caryophyllene epoxide greater than 0.70 mg/g and those of caryophyllene greater than 7.0 mg/g significantly deterred the harvest of test flakes in both cases. By testing this range of concentrations, a minimum deterrent concentration has been established, and it is three- to fourfold lower than

deterrent concentrations previously reported (Hubbell et al., 1983). Thus the number of plant species containing concentrations of caryophyllene and/or caryophyllene epoxide meaningful to leafcutters may be greater than previously supposed.

Within the Attini, primitive genera culture fungus on feces and detritus, while more advanced genera show an increasing reliance on living plant tissues for fungus culture (Weber, 1972). In this study, the plant natural products caryophyllene and caryophyllene epoxide showed similar but not identical effects on the behavior of *Atta* and *Acromyrmex*, the two most advanced attine genera. It would be of great interest to know whether members of more primitive attine genera are equally deterred by these compounds or if deterrency is correlated with the use of plant material in fungus culture. Bioassays using these two compounds at constant concentrations may provide a useful standard for comparative studies of other attine genera and may offer insights into the evolution of fungus culture in this group of insects.

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ALLELOPATHIC EFFECT OF ALFALFA (*Medicago sativa*) ON BLADYGRASS (*Imperata cylindrica*)

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Abstract—Greenhouse and laboratory experiments were conducted at the Agricultural and Water Resources Research Center Station, Baghdad, in 1985 and 1986 to investigate the possible allelopathic potential of alfalfa (*Medicago sativa* L.) and its decomposed residues on bladygrass (*Imperata cylindrica* L. Beauv.), a noxious weed in Iraq, and to isolate, characterize, and quantify possible allelopathic agents in alfalfa residues and root exudates. Results indicated that decomposed alfalfa roots and their associated soil produced a 51–56% reduction in bladygrass seed germination. Root and shoot length of bladygrass seedlings were reduced by an average of 88%. Decayed and undecayed mixtures of alfalfa roots and soil at 0.015:1 (w/w) inhibited bladygrass seedlings reproduced from rhizomes by 30 and 42%. It was found that root exudates of alfalfa seedlings caused significant reduction in shoot and root dry weights of bladygrass seedlings when alfalfa and bladygrass were grown together in nutrient culture. Caffeic, chlorogenic, isochlorogenic, *p*-coumaric, *p*-OH-benzoic, and ferulic acids were detected in alfalfa root exudates and residues. The highest amount (126 μ g phenolic acids/g soil) of these compounds was found in alfalfa root residues after six months of decomposition in soil.

Key Words—Allelopathy, *Medicago sativa*, *Imperata cylindrica*, root exudates, residues, decomposition.

INTRODUCTION

The term allelopathy includes the direct or indirect harmful and beneficial effects by one plant on another through the production of chemical compounds that escape into the environment (Rice, 1984). Studies concerning the manipulation of allelopathy of crops to control weeds have been increased recently. Al-Saa-

dawi et al. (1986) found that some *Sorghum bicolor* cultivars possess potential biological control of *Amaranthus retroflexus* by reducing its growth through root exudations. Root secretions of *Zea mays* L. inhibited the growth of common lambsquarters (*Chenopodium album* L.) and redroot pigweed (Dzubenko and Petrenko, 1971). Overland (1966) showed that allelopathy was involved in the mechanism of weed control by barley (*Hordium* spp.), wheat mulch, and rye (*Secale cereale* L.). Putnam and Duke (1974) found that some accessions of cucumber (*Cucumis sativus* L.) were able to inhibit proso millet (*Panicum miliaceum* L.) growth. Studies by Fay and Duke (1977) indicated that some accessions of (*Avena* spp.) contained scopoletin, an allelopathic agent. Germination and growth of wild mustard (*Brassica kaber*) were inhibited by aqueous leaf extracts of sunflower (*Helianthus annuus*) (Leather, 1983). Some soybean cultivars also possess the ability to inhibit growth of companion weeds by allelopathic exudates (Rose et al., 1984).

Bladygrass (*Imperata cylindrica* L. Beauv.), an upright rhizomatous grass, is widespread throughout the tropics and subtropics and is considered one of the worlds worst weeds (Holme et al., 1977). In Iraq, bladygrass is a noxious weed that spreads widely and infests almost all aspects of agriculture. Al-Juboory (1978) reported that some newly established citrus orchards have been abandoned by farmers due to heavy bladygrass infestation. Our preliminary field observations showed that alfalfa (*Medicago sativa*) was an excellent smother crop to bladygrass and in some instances was able to halt bladygrass seedling emergence for periods ranging between two and four years. The ability of alfalfa to smother bladygrass could be due mainly to physical competition (light and nutrients). However, alfalfa has been reported to contain water-soluble substances toxic to plants. Lawrence (1962) found that alfalfa roots contained water-soluble substances toxic to several grasses. Another study by Guenzi et al. (1964) indicated that extracts of alfalfa contained water-soluble substances that reduced shoot and root growth of corn seedlings. A recent study by Miller (1983) demonstrated the autotoxicity of alfalfa on the reestablishment of subsequent plantings. Therefore, the ability of alfalfa to compete strongly with bladygrass might not be due to physical competition only but also to allelopathy. This study was undertaken to (1) determine if residues of alfalfa roots in soil after different periods of decaying have allelopathic effects on bladygrass seedling growth, (2) determine the effects of alfalfa root exudates on bladygrass seedling growth, and (3) isolate and characterize possible allelopathic agents in alfalfa residues and root exudates.

METHODS AND MATERIALS

Effect of Decayed Alfalfa Roots on Bladygrass Seed Germination and Seedling Growth. A 1-year-old established alfalfa field was chosen to collect alfalfa roots and the surrounding soil in August 1985. The site was located 80 km north

of Baghdad at the Ishaki Research Station. The roots were separated from the soil, and both were air-dried for three days at an average temperature of 45°C, then ground to pass a 0.5-mm sieve, and mixed thoroughly at a ratio of 0.015:1 (w/w root to soil). The ratio was selected on the basis of actual weight of dead alfalfa secondary roots and the soil in their vicinity. The mixtures were placed in 30 × 10 × 10-cm plastic containers under greenhouse conditions and left to decay for 0, 3, and 6 months. The containers of the last two treatments (3 and 6 months decay periods) were watered with 500 ml of water every three days. Alfalfa-root-free soil collected from adjacent locations was used as a control. Ground peatmoss was mixed with the soil at a ratio of 0.015:1 (w/w peatmoss to soil) to substitute for alfalfa roots in the control pots. The average pHs of the root-soil and peatmoss-soil mixtures were 7.8 and 7.3, respectively.

Bladygrass inflorescence were collected from the same site during June 1985. The seeds were separated and stored at room temperature for germination tests. One hundred seeds were planted in 12-cm Petri dishes filled with a 100-ml volume of the previously prepared alfalfa root and peatmoss-soil mixtures. Ten Petri dishes were used for each treatment. Watering was done by adding 20 ml of tap water to each dish. After 10 days of incubation at 30°C, germination and length of radicles and shoots of bladygrass seedlings were recorded. Data were analyzed for significant differences by randomized complete-block design analysis of variance and Duncan's multiple-range test at the 5% level of significance.

Effect of Decayed Alfalfa Roots on Bladygrass Rhizomes Growth. To determine the effect of decayed alfalfa roots on bladygrass rhizome growth, the following planting media were prepared: (1) undecayed mixture of alfalfa roots with their planting soil at ratio of 0.015:1 w/w root to soil; (2) undecayed alfalfa-planted soil; (3) undecayed bare soil; (4) mixture of decayed alfalfa roots with their planting soil at a ratio of 0.015:1 (w/w roots to soil); (5) decayed alfalfa planting soil; and (6) decayed bare soil. Alfalfa-planted soil and roots were taken from a 1-year-old alfalfa field, while bare soil was collected from an adjacent field. All decayed planting media were prepared by placing 2 kg of ground and mixed soil mediums in 20-cm-diameter plastic pots and watered periodically to maintain them at field capacity for three months.

Bladygrass rhizomes were collected from heavily infested bladygrass sites located 80 km north of Baghdad in the fall of 1985. Rhizomes were cut into 4- to 5-cm segments with three nodes. Three uniform segments were selected and planted 3-4 cm deep in the planting mediums. Pots were placed in a greenhouse at an average 50-60% relative humidity and 25°C and watered periodically as required. Three replications of each treatment were used. After 20 days, all bladygrass nodes of the control treatment seedlings were counted, and the percent of seedling emergence was calculated based on that of the control treatment. Seedlings were counted, the lengths of roots and shoots were measured, and seedling dry weights noted. Data were analyzed for statistical differences by Duncan's multiple-range test with 5% level significant.

Effect of Alfalfa Root Exudates on Bladygrass Seedling Growth. This experiment was conducted to determine the ability of alfalfa seedlings to produce allelopathic inhibitors that may influence bladygrass seedling growth in the absence of physical competition. The procedure followed that described by Al-Saadawi and Rice (1982). Uniformly sized alfalfa and bladygrass seedlings were selected from seedlings grown in sand culture under greenhouse conditions at 25°C, 60% relative humidity, and 14 hr light. The average stem length of both seedlings was 7 ± 0.5 cm, with an average root length of 4 ± 0.5 cm. Roots were washed with tap water to remove sand. One seedling was placed in each end of a U-shaped Pyrex tube, 2.5 cm in diameter and 25 cm long. The seedlings were held in place by a stopper bored to admit the seedling stem, which was encased in cotton. The tube was wrapped with aluminum foil and filled with Hoagland's solution (Hoagland and Arnon, 1950). Three paired seedling treatments were used in this experiment: alfalfa-bladygrass, alfalfa-alfalfa, and bladygrass-bladygrass. Tubes were aerated daily by vacuum for 10 min, and the solutions were maintained at the same level by adding the required volume of solution. Each treatment was replicated 10 times. After 21 days, shoot and root dry weights of both seedlings were taken. Data were subjected to analysis of variance and LSD with a 5% level of significant.

Isolation and Characterization of Allelochemicals. Since phenolic compounds have been implicated in most allelopathic studies (Rice, 1984; Guenzi and McCalla, 1966; Putnam and Duke, 1974; Blum et al., 1985), we investigated their presence in alfalfa. The procedure used to isolate and identify the phenolic compounds was the same as described by McPherson et al. (1971). One hundred grams of the decomposed alfalfa-planted soil medium was mixed with 150 ml of 95% EtOH and sufficient 2 N NaOH to bring the pH to 11. The mixture was shaken for 48 hr using a mechanical shaker with periodic readjustment of pH to 11 and then the mixture was filtered under vacuum.

The filtrate was reduced to about 50 ml in vacuo at 50–60°C, centrifuged, and the supernatant brought to pH 7 with 2 N HCl. The resultant liquid was evaporated to dryness on a hot plate (60–65°C). The residue was ground to a powder, taken up into three washes of ether, acidified to pH 2 with 2 N HCl, and evaporated to dryness at room temperature under a fume hood. The residue was dissolved in 3 ml of 95% EtOH. The solution was applied to 2 × 30-cm strips of Whatman No. 1 chromatographic paper, developed in *n*-butanol–acetic acid–water (BAW, 63:10:27 v/v/v), and inspected with short- and longwave UV. Compounds were marked under UV light and eluted with 95% ethanol. The elutes were reduced to dryness, taken up in 3 ml absolute EtOH, and chromatographed on Whatman No. 1 paper in three solvent systems: BAW, 6% acetic, and IBW (isopropanol–*n*-butanol–water; 140:20:60 v/v/v). The R_f values in various solvent systems, colors under UV light, and colors in various reagents were determined for all detected compounds. Allelochemicals of alfalfa root exudates in the paired seedling experiment of alfalfa-alfalfa, alfalfa-blad-

ygrass, and bladygrass–bladygrass were isolated and characterized by collecting a total of 1750 ml of the growing solution in each U-tube after 20 days of growth, vacuum dried under 40°C, and then extracted and chromatographed by the previously mentioned procedure. Identification of the phenolic acids in the samples was made by comparing their R_f values and colors with those of known standards. Quantitative estimation of the total phenolic compounds for each decay period and exudate treatment was done using Folin-Dennis reagent according to Pearson (1970).

Biological Activities of Isolated Phenolic Compounds. The biological activity of all isolated allelochemicals was determined using bladygrass seeds. All distinctive bands resulting from the previous identification were cut out, placed on 9-cm-diameter Petri dishes containing 10 ml buffer, 6.8 pH, and 50 bladygrass seeds. Similar sized areas from blank chromatograms were used as a control. The Petri dishes were placed in a growth chamber with a 14-hr photoperiod and 30°C temperature. The test and control treatments were replicated three times. Seed germination and radicle and hypocotyl lengths were taken after 10 days of incubation.

RESULTS

Bladygrass Seed Germination and Seedling Growth. The percentage of bladygrass seed germination and seedling root and shoot length as affected by decomposed alfalfa roots and soil residue are summarized in Table 1. Germination percentages of bladygrass seeds were significantly decreased by incorporation alfalfa roots with planting soil and for the two periods of decomposition (3 and 6 months). As the time of decomposition increased, the germination decreased. The greatest reduction in seed germination (55%) resulted when a

TABLE 1. INFLUENCE OF DECOMPOSED ALFALFA ROOTS AND SOIL RESIDUES ON BLADYGRASS SEED GERMINATION AND SEEDLING ROOT AND SHOOT LENGTH^a

Treatments	Time of decomposition (months)	Germination (%)	Root length (mm)	Shoot length (mm)
Control (bare soil)	0	54.5 a	17 a	53 a
Alfalfa root and soil residue	0	40.4 b	3 b	15 b
Control (bare soil)	3	55.0 a	17 a	53 a
Alfalfa root and soil residue	3	30.0 c	1 c	12 b
Control (bare soil)	6	56.0 a	19 a	52 a
Alfalfa root and soil residue	6	25.0 d	1 c	7 c

^aMeans followed by the same letter are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

mixture of alfalfa roots and soil were subjected to 6 months of decomposition prior to germination tests. This treatment resulted in 25% germination compared to 56% for the control (bare soil) treatment.

Bladygrass root and shoot lengths were significantly reduced by alfalfa root and soil residues. The percentage reduction in root length was 92 and 95% for the 3 and 6 months periods of decomposition, respectively. The same trend on shoot length was detected, whereas higher reductions resulted with the 6-month period of decomposition. Incorporation and decomposition of alfalfa roots with soil reduced shoot length to 7 cm compared to 52 cm for the control treatment.

Effect of Decayed Alfalfa Roots on Bladygrass Rhizomes Growth. The percentages of bladygrass seedling emergence, radicle and shoot length, and seedling dry weight as affected by decayed alfalfa roots and soil are summarized in Table 2. Seedling emergence was significantly reduced by undecayed and decayed alfalfa roots and soil planting mediums. Length of time seemed to have no significant effect on bladygrass seedling emergence, because no differences were detected between the 0- and 3-month treatments. Both periods inhibited emergence to 30–42% compared to the control treatment (bare soil).

Bladygrass radicle length was greatly inhibited by all treatments of decayed or undecayed alfalfa root–soil mixtures or by alfalfa residue. Percentage of inhibition was found to be 95 and 97% for the undecayed and decayed root–soil, respectively. Time of decomposition had no significant effect on the inhibition of radicle length. However, the 3-months alfalfa root–soil decomposition resulted in the shortest radicle (1 mm).

Similar trends of effect on bladygrass shoot elongation were noticed. A higher percentage of shoot length inhibition resulted from the 3-month decay

TABLE 2. INFLUENCE OF DECOMPOSED ALFALFA ROOTS AND SOIL RESIDUES AT DIFFERENT PERIODS OF DECOMPOSITION ON GROWTH OF BLADYGRASS SEEDLINGS PRODUCED FROM RHIZOMES^a

Treatment	Time of decomposition (months)	Seedling emergence (%)	Radicle length (mm)	Shoot length (mm)	Dry weight (mg/seedling)
Bare soil (control)	0	100 a	32 b	34 b	11 b
Alfalfa root and soil residue	0	30 b	2 c	4 c	3 d
Alfalfa-planted soil	0	30 b	2 c	8 c	4 d
Bare soil (control)	3	100 a	40 a	45 a	21 a
Alfalfa root and soil residue	3	30 b	1 c	3 c	2 d
Alfalfa-planted soil	3	42 b	3 c	3 c	3 c

^aMeans followed by the same letter are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

treatments than that of the undecayed. Incorporation of alfalfa roots with soil and decomposed for 3 months reduced shoot length to an average of 94% from that of the check treatment. The dry weight of bladygrass seedlings was also significantly reduced by alfalfa residues. The highest percentage of inhibition (90%) was obtained from the 3-month decayed of alfalfa root-soil planting medium.

Effect of Alfalfa Root Exudates on Bladygrass Seedling Growth. The influence of root exudates on the dry weight of alfalfa and bladygrass seedling shoots and roots is summarized in Table 3. Bladygrass seedling shoot dry weight grown with alfalfa was significantly reduced. The percentage of reduction in bladygrass shoot dry weight was 42% compared to its dry weight in the bladygrass paired seedling treatment. Great reductions in the dry weights of both alfalfa—seedling shoots resulted when they were grown together.

The root dry weight of bladygrass was less when grown with alfalfa. This was significantly different from that of the control treatment (bladygrass—bladygrass). The dry weight of alfalfa roots in the alfalfa—alfalfa paired seedling treatment was also inhibited by 20%; however, this reduction was less than that for the shoot dry weight.

Isolation and Characterization of Allelochemicals. Chromatography of alfalfa root residues and exudates (Table 4) showed the presence of phenolic acids: caffeic, chlorogenic, isochlorogenic, *p*-coumaric, *p*-OH-benzoic, and ferulic. In addition, quercetin, one of the flavinoid group, and four unknown compounds were present. The R_f values under different solvents, their fluorescences under long and short UV with and without ammonia, and their colors

TABLE 3. INFLUENCE OF ALFALFA ROOT EXUDATES ON BLADYGRASS SHOOT DRY WEIGHT^a

Treatment	Shoot dry weight (mg/seedling)	Root dry weight (mg/seedling)
Alfalfa + bladygrass	153	20
Alfalfa + alfalfa	45	5
Alfalfa + bladygrass	83	15
Bladygrass + bladygrass	86	17
	79	32
	76	30
LSD ($P \leq 0.05$)	26	21

^aNumber represents the average weight of 10 seedlings.

TABLE 4. CHROMATOGRAPHY OF EXTRACTS FROM DECOMPOSED ALFALFA-PLANTED SOIL AND ROOT EXUDATES OF ALFALFA AND BLADYGRASS

Compound ^a	<i>R_f</i> on Whatman No. 1 ^b				Fluorescence ^c						Reagent color ^c	
	6% AA	BAW	IBW	- NH ₃	Long UV		Short UV		Sulfanilic acid	<i>p</i> -Nitroanilin	FeCl ₃	K ₃ Fe(CN) ₆
					+ NH ₃	+ NH ₃	- NH ₃	+ NH ₃				
Caffeic acid	0.73	0.68	0.66	blu	1 blu	blu	1 blu	1 blu	—	bn bk	blu	blu
Isochlorogenic acid	0.24	0.88	0.85	1 blu	f blu gr	1 blu	1 blu	f blu gr	yel-tan	bn	blu	blu
Chlorogenic acid	0.63	0.71	0.68	1 blu	f blu gr	1 blu	1 blu	f blu gr	yel-tan	bn	blu	blu
<i>p</i> -Coumaric acid	0.60	0.82	0.86	pur	1 blu	pur	1 blu	1 blu	or-red	bn bk	blu	blu
<i>p</i> -OH-benzoic acid	0.83	0.75	0.69	pur abs	pur	pur abs	pur	pur	or-red	f win	f yel gr	f yel gr
Ferulic acid	0.8	0.9	0.67	bl	1 blu	blu	1 blu	1 blu	tan	f bn bk	d blu	d blu
Quercetin	0.01	0.96	0.76	yel	yel	yel	yel	yel	bn	tan-yel	d blu	d blu
Unknown 1	0.02	0.94	0.92	1 blu	blu-gr	1 blu	1 blu	blu-gr	bn	bn bk	blu	blu
Unknown 2	0.09	0.92	0.91	yel-bn	yel-bn	yel-bn	yel-bn	yel-bn	f bn	yel-bn	blu	blu
Unknown 3	0.05	0.98	0.96	bn	red-bn	bn	bn	red-bn	f bn	yel-bn	blu	blu
Unknown 4	0.10	0.98	0.81	blu	blu	blu	blu	blu	f or	f pur	blu	blu

^aCaffeic, chlorogenic, isochlorogenic, *p*-OH-benzoic acids, and unknown 2 were detected in alfalfa soil residue and root exudates; Ferulic acid, quercetin, and unknown 4 in soil residues only; *p*-coumaric acid and unknown 1 in root exudates only.

^bEach *R_f* is the average of four runs.

^cabs = absorption, bk = black, bn = brown, d = deep, f = faint, gr = green, l = light, or = orange, pur = purple, yel = yellow, and blu = blue.

TABLE 5. AMOUNT OF PHENOLIC ACIDS IN ALFALFA ROOT AND SOIL RESIDUES FOR DIFFERENT DECOMPOSITION PERIODS

Treatment	Time of decomposition (months)	Ethanol extract ($\mu\text{g/g}$ soil)	Hexane extract ($\mu\text{g/g}$ soil)	Total ($\mu\text{g/g}$ soil)
1	0	4	31	35
2	3	32	49	81
3	6	72	54	126

with various reagents are shown in Table 4. The R_f values and colors of the unknown detected compounds 1, 2, 3, and 4 suggest that they might be flavinoids.

Quantitative estimation of the phenolic compounds in decomposed alfalfa residues is summarized in Table 5. The cumulative amounts of the phenolic acids in the ethanol and hexane extracts showed that longer periods of decomposition resulted in higher accumulation of phenolic compounds. The 6-month period of decay resulted in 126 μg of phenolics/g of soil in comparison to 81 $\mu\text{g/g}$ in the 3-month decomposition. Root exudates of alfalfa-bladygrass paired seedlings was found to contain 12 μg phenolics/ml of analyzed growing solution while that of alfalfa-alfalfa and bladygrass-bladygrass seedlings contained 2.1 and 1.4 $\mu\text{g/ml}$ of solution, respectively.

Biological Activities of Isolated Allelochemicals. Percentages of bladygrass seed germination and seedling root and radicle length as affected by isolated phytotoxins or their mixtures are summarized in Table 6. Bladygrass seed germination was significantly inhibited by all isolated compounds or their combinations in comparison to the control treatment. Isochlorogenic, chlorogenic, ferulic acids, and the mixtures of the isolated compounds from the 6-month decayed alfalfa residues resulted in inhibition. Shoot and radicle lengths of bladygrass seedlings were also inhibited by all isolated phenolic acids and their combinations.

DISCUSSION

The results of these experiments show that alfalfa soil residues reduced germination and seedling growth of bladygrass. It was found that the longer the period of decay, the higher the inhibition of seed germination. Decomposition of alfalfa roots for 6 months reduced seed germination by 55%. Seedling growth (shoot and root length) of bladygrass was also inhibited by incorporation and

TABLE 6. EFFECT OF ALFALFA ISOLATED PHYTOTOXINS ON BLADYGRASS SEED GERMINATION AND SEEDLING SHOOT AND RADICLE LENGTH^a

Phytotoxin	Germination (%)	Shoot length (mm)	Radicle length (mm)
Caffeic acid	25.0	1	1
Isochlorogenic acid	8.0	1	0.3
Chlorogenic acid	10.0	1	1
Ferulic acid	11.0	1	0.1
<i>p</i> -OH-Benzoic acid	22.0	1	0.1
<i>p</i> -Coumaric acid	27.0	2	1
Quercetin	25.0	1	0.1
Unknown 1	24.3	2	0.1
Unknown 2	20.3	1	1
Unknown 3	27.7	1	1
Unknown 4	14.3	0.5	0.1
Combination of compounds ^b	42.7	5	3
Combination of compounds ^c	18.7	5	1
Combination of compounds ^d	8.0	3	1
Control	51.3	8	5
LSD ($P \leq 0.05$)	4.4	0.6	4

^a Values of three replications.

^b Compounds isolated from 0-month decay period.

^c Compounds isolated from 3-month decaying of alfalfa residues.

^d Compounds isolated from 6-month decay period.

decomposition of alfalfa roots. The reduced seed germination and seedling growth of bladygrass by alfalfa residues was apparently due to its allelopathic effect. This is supported by the fact that the decayed and undecayed bare soil (control) planting media did not inhibit the germination and growth of bladygrass (Table 1). No previous work has been published on the allelopathic effect of alfalfa on bladygrass. However, Nielsen et al. (1960) showed that alfalfa, among other crops, caused the greatest delay in seed germination and seedling growth of corn, soybean, peas, and oats. Another study by Guenzi et al. (1964) supports the previous observation of the allelopathic effect on corn. They demonstrated that this effect was due to saponins.

Bladygrass seedling growth emerging from rhizomes was also inhibited by alfalfa residues. Significant reduction occurred in percent of bladygrass seedling emergence, their radicle and shoot length, and their cumulative dry weights (Table 2) from alfalfa residues. Decomposing alfalfa roots appear to produce greater inhibition than undecomposed roots. This may be due to a higher release of phytotoxic substances. However, this conclusion may not be definite since

TABLE 7. AMOUNTS OF PHENOLIC ACIDS IN ROOT EXUDATES OF ALFALFA AND BLADYGRASS PAIRED SEEDLINGS

Treatments	Cumulative amounts of phenolic acids ($\mu\text{g/ml}$ growing medium)
Alfalfa + alfalfa	2.1
Alfalfa + bladygrass	12
Bladygrass + bladygrass	1.4

other factors such as microbial action might play a role (Kommedahi et al., 1959).

Inhibition of bladygrass seedling root and shoot dry weights by alfalfa root exudates in the absence of physical competition (Table 3) is another indication that the reduction is due to allelochemicals exuded from alfalfa. This is supported by previous work by Nielsen et al. (1960) and Guenzi et al. (1964), who demonstrated that alfalfa contains water-soluble materials that inhibit seedling growth of several crops. Miller (1983) has established the autotoxicity of alfalfa. He observed that phytotoxic factors released from alfalfa affect the reestablishment of alfalfa. A later study by Tsuzuki and Kawagoe (1984) showed also that root exudates from alfalfa reduced the dry weight of barley, alfalfa, and radish seedlings.

The isolation and identification of caffeic, isochlorogenic, chlorogenic, *p*-coumaric, *p*-OH-benzoic, and ferulic acids and quercetin flavinoid from alfalfa root residues and exudates also support the potential for allelopathic interactions of alfalfa on bladygrass. Most phenolic compounds have been reported as being strong allelopathic agents (Fay and Duke, 1977; Rice, 1984; Blum et al., 1985; Putnam and Duke, 1974; Abdul-Rahman and Al-Naib, 1986). Our results (Table 7) showed that these isolated phenolic acids were biologically active by reducing bladygrass seed germination and seedling root length. This indicates that the allelopathic effect of alfalfa on bladygrass may be due to the presence of these phenolic acids.

It is clear from these results that the reduction in bladygrass seedling growth was due to allelochemicals released from alfalfa. This probably explains the ability of alfalfa to act as a strong smother crop to bladygrass (personal field observations). However, the ability of alfalfa to compete strongly with bladygrass for light, space, and nutrients might enhance the smothering ability of alfalfa in the field. The results of this study could be an added support to previous studies by many researchers (Rose et al., 1984; Leather, 1983; Fay and Duke, 1977) to manipulate the allelopathic phenomenon of some crops to control weeds.

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CUTICULAR HYDROCARBONS OF THE SCREWWORM,
Cochliomyia hominivorax (DIPTERA: CALLIPHORIDAE)
Isolation, Identification, and Quantification as a Function of
Age, Sex, and Irradiation¹

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Abstract—A novel series of 2,X-dimethylalkanes were isolated and identified. The nonpolar fraction of the surface lipids secreted by the adult (5-day-old) screwworm, *Cochliomyia hominivorax*, contains over 130 different hydrocarbons comprising normal alkanes (32% of the total hydrocarbon), branched alkanes (53%), and monoalkenes (11%). Branched alkanes included monomethylalkanes with substitution in all possible positions except for 4-methylalkanes, internally branched dimethylalkanes, and 2,X- and 3,X-dimethylalkanes. At emergence, adults of both sexes of the 009 strain have nearly identical gas chromatographic profiles, which diverge as the insect ages. Irradiation of pharate pupae does not affect the hydrocarbon produced.

Key Words—Insect, screwworm, *Cochliomyia hominivorax*, Diptera, Calliphoridae, cuticular hydrocarbons, mass spectrometry, 2,X-dimethylalkanes.

INTRODUCTION

The screwworm, *Cochliomyia hominivorax*, belongs to the family of blowflies (Diptera: Calliphoridae). The female oviposits in open sores and wounds of mammals, and the larvae, after hatching, feed on the living flesh surrounding

¹ Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the USDA.

the wound (myiasis). The insect has been a pest of the livestock industry in the United States since the turn of the century. A "barrier" of released radiation-sterilized insects is now located in southern Mexico at the isthmus of Tehuantepec. The insect is considered to be officially eradicated north of the barrier in Mexico. The screwworm was colonized at Fargo, North Dakota, in 1975 from a culture of the 009 strain from the screwworm facility in Mission, Texas. The 009 strain is a composite formed with screwworms collected from 28 different Texas locations.

The purpose of this study was to gather information of the screwworm's nonpolar surface lipid composition as a base for current and future studies in such areas as: the effect of sterilizing radiation on quantity and quality of hydrocarbon production, which may negatively affect the sterile release program, lipid biosynthesis, the role these lipids have on the behavioral functions (pheromone), and as a possible chemotaxonomic indicator for strains from various geographical locations.

We had earlier reported the gas chromatographic composition profiles of surface lipid extracts from screwworms from different geographical locations (Pomonis and Mackley, 1985). We now report, for the 009 strain, the isolation and characterization of the hydrocarbon fraction of the surface lipids, the quantification of the *n*-alkanes and methylalkanes, and the relationships of these quantities to age, sex, and effect of 6000 rads of gamma irradiation.

METHODS AND MATERIALS

Biological. Insects were reared as described by Hammack (1984) and were of the 009 strain, which had been in laboratory culture for nearly 10 years at Fargo. The insects were separated by sex 24 hr after emergence and reared separately for use at the appropriate age. Pupae were irradiated at four days preemergence in a cobalt-60 source with 6000 rads and separated by sex within 24 hr postemergence; experimental data were compared to those from nonirradiated pupae.

Extraction of Lipids. Flies, which were killed by freezing, were counted and weighed as a single mass with the number of flies as the denominator in the standardization of quantities (Jackson and Bartelt, 1986). The surface lipids were extracted with CH_2Cl_2 , 3×35 ml/45 insects for 1 min. For the screwworm, extraction periods greater than 5 min extracted internal lipids that appeared as a broad peak in the gas chromatogram at Kovats retention index (KI; Kovats, 1965) of about 1700–1900. The solvent was removed in vacuo, and 3-methylnonadecane and 3-methylheneicosane (KI 1975 and 2175, respectively, and 100 μg each per 45 insects) were added as internal standards for chromatography. Silica column chromatography separated the hydrocarbon fraction from the other lipids. The alkenes were separated from the alkanes by

argentation thin-layer chromatography (TLC) or argentation column chromatography. The methyl alkanes were separated from the *n*-alkanes by molecular sieving (O'Connor et al., 1962).

Alkene Derivatization. Alkenes were derivatized as the methoxy adducts for analyses by gas chromatography-mass spectrometry (GC-MS) using the methoxy-mercuration reaction described in the literature (Abley et al., 1970; Plattner et al., 1976; Blomquist et al., 1980).

Gas Chromatography (GC). Analyses were performed with a Varian model 3700 flame ionization instrument fitted with a multipurpose heated (225°C) variable split injector (100:1) and a cross-linked methylsilicone fused silica capillary column [Hewlett-Packard (H-P) 12 m × 0.20 mm ID, film = 0.33 μm, He carrier gas at 0.77 ml/min (30 cm/sec) at a head pressure of 12.5 psig]. The GC was programmed from 150 to 320°C at a variable rate (usually at 4 or 2°/min) with a 2-min initial and a 15-min final isothermal hold. Chromatographic data were reported by a H-P model 3390A integrator interfaced to the GC. For quantification and to determine the KI, a mixture of nearly equal and exactly weighed quantities of (ca. 1.0 μg) each of the normal alkanes: C₂₀H₄₂, C₂₄H₅₀, C₂₈H₅₈, C₃₂H₆₆, C₃₆H₇₄, C₄₀H₈₂, and C₄₄H₉₀ was used.

Gas Chromatography-Mass Spectrometry (GC-MS). The GC-MS analyses were performed by means of a modified Varian model 3700 packed column gas chromatograph coupled to Finnegan-Mat 112S mass spectrometer in the electron impact mode and fitted with a Carter-Cook jet separator. For chromatography, a 3.05-m × 0.635-cm (OD) glass column was packed with 3.5% OV-101 on Gas-Chrom Q, 100-120 mesh and temperature programmed from 150 to 324°C at 4°/min, using He carrier gas at 25 ml/min. Alternatively, for some GC-MS analyses, an H-P 5790 GC with a standard H-P cool on-column injector was used; this was fitted with a 12.5-m cross-linked methylsilicone fused silica capillary column whose exit inserted directly into the ionizing chamber of the 112S spectrometer. The GC was programmed with a 2-min initial isothermal hold at 60°C, heated to 150°C at 28°/min, then from 150 to 325°C at 3°/min with helium as carrier gas at 1 ml/min.

Mass spectra of the methylalkanes were interpreted according to the criteria proposed by McCarthy et al. (1968), Nelson and Sukkestad (1970), Nelson et al. (1972), and Pomonis et al. (1978, 1980). Mass spectra of the alkene methoxy derivatives were interpreted by criteria proposed by Blomquist et al. (1980).

RESULTS

Table 1 summarizes the effect of age, sex, and irradiation on the quantities of various cuticular lipid fractions produced by the screwworm. Total surface lipid increased as the fly aged for each type of treatment (column 1). Hydro-

TABLE 1. EFFECT OF AGE, SEX, AND PUPAL IRRADIATION ON AMOUNT OF TOTAL SURFACE LIPIDS, *n*-ALKANES, METHYLALKANES, AND ALKENES SYNTHESIZED BY ADULT SCREWORM^a

	Total surface lipid ($\mu\text{g}/\text{fly}$) ^b	<i>n</i> -Alkanes ($\mu\text{g}/\text{fly}$) ^b	Methylalkanes ($\mu\text{g}/\text{fly}$) ^b	Alkenes ($\mu\text{g}/\text{fly}$) ^b
Newly emerged				
Female				
Control	53 \pm 15	15 \pm 6	17 \pm 6	2 \pm 1
Irradiated	56 \pm 8	12 \pm 3	18 \pm 4	5 \pm 3
Male				
Control	50 \pm 12	11 \pm 5	22 \pm 9	5 \pm 5
Irradiated	52 \pm 9	7 \pm 3	22 \pm 3	2 \pm 1
3-Day-old				
Female				
Control	74 \pm 6	18 \pm 4	29 \pm 4	10 \pm 6
Irradiated	84 \pm 11	18 \pm 2	37 \pm 2	10 \pm 5
Male				
Control	86 \pm 12	18 \pm 5	25 \pm 3	10 \pm 3
Irradiated	57 \pm 22	18 \pm 2	32 \pm 6	16 \pm 15
5-Day-old				
Female				
Control	132 \pm 61	21 \pm 4	36 \pm 8	11 \pm 7
Irradiated	121 \pm 40	23 \pm 10	40 \pm 12	10 \pm 4
Male				
Control	114 \pm 43	17 \pm 3	30 \pm 2	8 \pm 6
Irradiated	89 \pm 11	16 \pm 4	31 \pm 3	13 \pm 9

^aFargo 009 strain $F > 100$.

^bMean \pm SD; 3 replicates; $N = 3 \times 45$.

carbons comprised a majority of the surface lipids for newly emerged (NE) adults (values discussed are for control female flies although values for both sexes are listed in Table 1). As the fly aged, the absolute amount of hydrocarbon increased from 34 to 50 to 74 μg , respectively, for the three ages. For all treatments in the hydrocarbon group, the quantity of *n*-alkanes remained nearly constant. The total quantity of the methylalkanes showed an increase as the insect aged for all treatments. The alkene fraction showed a high degree of variability and contained the lowest quantities of hydrocarbons of the three fractions considered. Irradiation of pharate pupae did not appear to affect synthesis of the lipids in adults. No significant differences in quantities or type of alkanes listed in Table 1 were observed between irradiated and nonirradiated insects and therefore are not included in the data of Table 2 (*t* test for paired observations with two degrees of freedom showed no significant difference; $n = 3 \times 45$).

Nearly 130 different alkanes were found (Table 2), including methylal-

TABLE 2. KOVATS INDICES (KI) AND QUANTITIES OF METHYLALKANES FROM ADULT SCREWORMS COMPARED BY AGE AND SEX^a

KI	Compounds	Newly emerged σ^b ($\mu\text{g}/\text{fly} \pm \text{SD}$)		3-Day-old ($\mu\text{g}/\text{fly} \pm \text{SD}$)		5-Day-old ($\mu\text{g}/\text{fly} \pm \text{SD}$)	
		σ	ρ	σ	ρ	σ	ρ
2200	<i>n</i> -Docosane					0.2 ^c	
2300	<i>n</i> -Tricosane	0.8 ^c				0.9 \pm 0.2	
2337	11-Methyltricosane		{			1.6 \pm 0.2	
2339	9-Methyltricosane	1.4 \pm 0.5					
2343	7-Methyltricosane	0.5 \pm 0.2				0.5 \pm 0.0	
2372	9,13-; 7,11-Dimethyltricosane	{	{			{	
2374	3-Methyltricosane			1.7 \pm 0.7			1.4 \pm 0.3
2400	<i>n</i> -Tetracosane	0.3 \pm 0.1				1.3 \pm 0.3	
2435-40	12-; 11-; 10-; 9-Methyltetracosane	0.8 \pm 0.4				0.5 \pm 0.1	
2500	<i>n</i> -Pentacosane	3.0 \pm 0.4		0.7 \pm 0.3		4.3 \pm 1.9	1.2 \pm 0.5
2537	13-; 11-; 9-Methylpentacosane	7.7 \pm 2.0	{	1.3 \pm 1.1		9.1 \pm 1.2	
2544	7-Methylpentacosane						
2556	5-Methylpentacosane	1.3 \pm 0.4				0.7 \pm 0.2	1.0 \pm 0.7
2572	11, 15-; 9,13-Dimethylpentacosane	1.4 \pm 0.6		0.4 \pm 0.3		1.2 \pm 0.2	
2574	3-Methylpentacosane	0.8 \pm 0.4				1.1 \pm 0.3	
2600	<i>n</i> -Hexacosane	0.4 \pm 0.2		0.1 ^c		0.4 \pm 0.1	0.5 \pm 0.3
2637	13-; 12-; 11-; 10-; 9-Methylhexacosane	0.7 \pm 0.1		0.4 \pm 0.3 ^d		0.7 \pm 0.4	
2670	11, 15-; 9,13-Dimethylhexacosane						
2700	<i>n</i> -Heptacosane	4.6 \pm 1.5	0.6 \pm 0.4	2.2 \pm 0.8		5.4 \pm 0.5	3.9 \pm 1.6
2735	13-; 11-Methylheptacosane	3.8 \pm 0.2		3.7 \pm 2.0		3.1 \pm 0.2	3.3 \pm 1.2
2739	9-Methylheptacosane						
2744	7-Methylheptacosane						
2752	5-Methylheptacosane						
2758	11, 15-; 9,13-Dimethylheptacosane			0.7 \pm 0.2			0.7 \pm 0.3
2773	3-Methylheptacosane	1.5 \pm 0.2	0.5 \pm 0.1	1.4 \pm 0.7		1.8 \pm 0.2	1.3 \pm 0.4
2800	<i>n</i> -Octacosane	0.4 \pm 0.3 ^d		0.1 ^c		0.4 ^c	1.8 \pm 0.5

TABLE 2. Continued

KI	Compounds	Newly emerged σ^b ($\mu\text{g}/\text{fly} \pm \text{SD}$)		3-Day-old ($\mu\text{g}/\text{fly} \pm \text{SD}$)		5-Day-old ($\mu\text{g}/\text{fly} \pm \text{SD}$)		
		σ	\bar{q}	σ	\bar{q}	σ	\bar{q}	
2835-40	14-, 13-, 12-, 11-, 10-, 9-, 8-Methyloctacosane	0.4 \pm 0.0	0.7 \pm 0.1				0.6 \pm 0.1	
2864	2-Methylotacosane	2.5 \pm 0.2	1.5 \pm 0.2	1.2 \pm 0.3 ^d		0.7 \pm 0.1	1.0 \pm 0.2	
2870	12, 16-, 10, 14-Dimethyloctacosane	$\left\{ \begin{array}{l} 1.0 \pm 0.2 \\ 0.3^c \\ 4.9 \pm 0.2 \end{array} \right.$	0.5 \pm 0.2				0.3 \pm 0.0 ^d	
2895-	2,20-, 2,18-, 2,16-, 2,14-, 2,12-,							
2905	2,10-, 2,8-, 2,6-Dimethyloctacosane							
2900	<i>n</i> -Nonacosane	4.9 \pm 0.2	5.0 \pm 0.7	4.0 \pm 0.7		4.3 \pm 0.4	6.7 \pm 0.3	
2936	15-, 13-, 11-Methylnonacosane	8.6 \pm 1.0	6.7 \pm 0.7	3.2 \pm 0.4		1.8 \pm 0.2	5.3 \pm 0.8	
2939	9-Methylnonacosane							
2944	7-Methylnonacosane	1.2 \pm 0.1	0.9 \pm 0.2				0.8 \pm 0.1	
2952	5-Methylnonacosane	0.7 \pm 0.1	0.7 \pm 0.1				0.6 \pm 0.1	
2963	13,17-, 11,15-Dimethylnonacosane	2.6 \pm 0.5	1.6 \pm 0.3	1.0 \pm 0.3		0.4 ^c	1.2 \pm 0.2	
2973	3-Methylnonacosane	1.8 \pm 0.2	1.9 \pm 0.3	1.0 \pm 0.2		0.9 \pm 0.3	1.5 \pm 0.3	
2995-	3,17-, 3,15-, 3,13-, 3,11-,	0.5 \pm 0.1						
3006	3,9-, 3,7-Dimethylnonacosane	0.8 \pm 0.1	0.4 \pm 0.1 ^d					
3000	<i>n</i> -Triacotane		0.6 \pm 0.2 ^d				0.4 \pm 0.1	
3035-	15-, 14-, 13-, 12-, 11-,	0.8 \pm 0.2						
3045	10-, 9-, 8-Methyltriacontane							
3065	2-Methyltriacontane	1.3 \pm 0.2	0.9 \pm 0.1	0.5 \pm 0.0		0.3 \pm 0.0	0.5 \pm 0.0 ^d	
3095-	2,20-, 2,18-, 2,16-, 2,14-,	0.8 \pm 0.1	0.4 \pm 0.0				0.7 ^c	
3105	2,12-, 2,10-, 2,8-, 2,6-Dimethyltriacontane	0.3 ^c						
3100	<i>n</i> -Henriacontane	1.7 \pm 0.5	6.7 \pm 0.9	0.8 \pm 0.4			1.1 \pm 0.2	
3130-45	15-, 13-, 11-, 9-, 7-Methylhentriacontane	1.4 \pm 0.2	2.7 \pm 0.8	0.7 \pm 0.1		0.5 \pm 0.0	2.2 \pm 0.6	
3155	5-Methylhentriacontane							
3158	13,17-, 11,15-, 9,13-, 7,11-Dimethylhentriacontane	0.9 \pm 0.2	1.2 \pm 0.1 ^d				1.0 \pm 0.0	
3175	3-Methylhentriacontane		0.5 \pm 0.4				0.6 \pm 0.1	
3200	<i>n</i> -Dotriacontane		0.8 \pm 0.1 ^d					

3230	16-, 15-, 14-, 13-, 12-Methylotriacontane			0.4 ± 0.2 ^d	0.3 ± 0.1
3300	<i>n</i> -Triacontane		0.2 ^c		
3330	17-, 15-, 13-, 11-, 9-Methyltriacontane	0.6 ± 0.2		3.2 ± 2.0	0.7 ± 0.0
3345	7-Methyltriacontane				0.4 ^c
3360	15, 19-, 13, 17-, 11, 15-, 9, 13-, 7-11-Dimethyltrtriacontane			2.4 ± 1.5	2.0 ± 0.1
3375	3-Methyltriacontane				0.3 ± 0.1 ^d
3430	17-, 16-, 15-, 14-, 13-Methyltetriacontane		0.7 ^c		0.4 ± 0.1 ^d
3460	14, 18-, 13, 17-, 12, 16-, 10, 14-Dimethyltetriacontane		0.6 ^c		0.4 ± 0.1 ^d
3530-40	17-, 15-, 13-, 11-, 9-, 7-Methylpentatriacontane	0.5 ± 0.2		1.5 ± 1.1	0.4 ± 0.2
3555	15, 19-, 13, 17-Dimethylpentatriacontane			1.8 ± 1.4	1.6 ± 0.7
					2.2 ± 1.0

^a Fargo 009 Strain, F₁₀₃ to F₁₀₉. Analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Only data from nonirradiated flies is shown since irradiated insects had similar values. Blank indicates no peak or that the recorder did not integrate or sense peak. Values are means from pooled samples where *N* = 3 replicates of 45 individuals per sample and each sample injected once.

^b Data for males and females identical except for male KI 2537.

^c GC peaks were sensed by integrator for one of three replicates.

^d GC peaks were sensed by integrator for two of three replicates.

^e Capillary GC-MS does not always resolve all isomers of a homologous series; however, KI were determined by GC alone (see Pomonis et al., 1989).

kanes with branching at nearly all possible positions as well as three different types of dimethylalkanes. The dimethyl alkanes include the well-documented internally branched compounds with three methylene units separating the methyl branches as well as the novel 2,X- and the 3,X-dimethylalkanes where X signifies the carbon carrying the second methyl group at 3, 5, 7, . . . methylene units of separation (Blomquist et al., 1987). Interpretation of the spectra of the methylalkanes is straightforward (Pomonis et al., 1978, 1980), while the interpretation of the 2,X-dimethylalkanes was based on information obtained from synthetic 2,X-dimethylalkanes reported in the companion paper (Pomonis et al., 1989).

The hydrocarbons were characterized by GC-MS and their KI values, names, and quantified values (averaged, from triplicate runs) of the chromatographic peaks (such as in Figure 1) are given in Table 2 for each of the three age groups of flies. The values for the quantities discussed below are for the control groups, but the trends were similar for the irradiated groups. Sexual dimorphism is evident from the chromatograms shown in Figure 1 for the three age groups. The two peaks at KI 1975 (3-methylnonadecane) and KI 2175 (3-methylheneicosane) are the internal standards and represent the detector response equivalent to 1 μg of material. The standard peaks are not exactly aligned because of operator differences; however, the chromatograms are aligned on KI 2936.

The *n*-alkanes were the second most abundant group of hydrocarbons found (Table 1). They ranged in size from 22 to 33 carbons with the odd-numbered homologs present in greater quantities than the even-numbered ones (Table 2). The male produced greater quantities of *n*-pentacosane than the female. The female produced greater quantities of *n*-hentriacontane. Both sexes showed increases with age for the *n*-heptacosane and a steady-state quantity for *n*-nonacosane.

The chromatographic profile of NE males and females in Figure 1 are similar except for the peak at KI 2537 (9-, 11-, and 13-methylpentacosane), which showed an increase in the amount with age, while in the female KI 2537 appeared at 3 days and diminished at 5 days of age. In males, the methylnonacosanes (KI 2936) decreased in quantity with age, while the methylheptacosanes (KI 2735 and 2773) increased. In females, the methyltrtriacontanes (KI 3331), methylpentatriacontanes (KI 3530), and 5-methylpentatriacontane (KI 3558) peaks showed increases beginning with day 3 and showed a threefold increase in quantity over the male with age.

The 2-methylalkanes were found in homologous even-numbered hydrocarbons, whereas the 3-methylalkanes were in homologous odd-numbered hydrocarbons. The quantities of 2-methyloctacosane (KI 2864) and 2-methyltricosane (KI 3065) decreased as both sexes aged. In males, the 3-methyltricosane, 3-methylpentacosane, and 3-methylheptacosane (KI 2374, 2574, 2773, respectively) showed an increase in quantity with age. However, the amounts

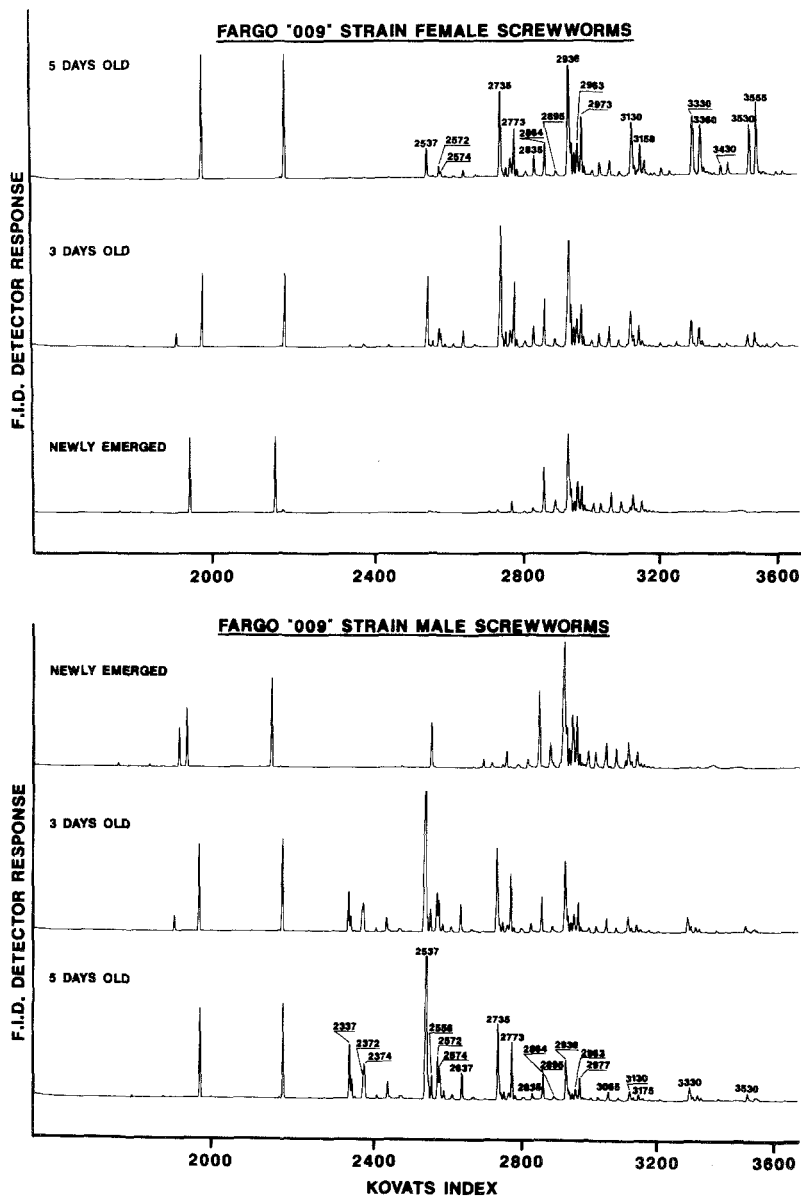


FIG. 1. Comparison of representative capillary gas chromatograms of the methylalkane fraction from surface extracts of screwworm adults (009 strain) by sex and age. Slight differences in each chromatogram do not allow for perfect alignment of all peaks; however, they are arbitrarily aligned on KI 2936 (methylnonacosanes). Peaks at KI 1975 (3-methylnonadecane) and KI 2175 (3-methylheneicosane) are those from standards added to the initial extracts and represent the detector response to $1 \mu\text{g}$ of each standard.

of the 3-methylnonacosane (KI 2977) showed a decrease with aging to half the original amount in NE. Sexual dimorphism was evident by the presence of additional higher 3-methylalkanes in the female surface lipids. Thus, 3-methylpentacosane, 3-methylheptacosane, 3-methylhentriacontane, and 3-methyltriacontane (KI 2372, 2773, 3175, and 3375, respectively) showed slight increases with age, whereas 3-methylnonacosane (KI 2973) remained nearly constant.

It was important to the interpretation of the mass spectra of the unknowns to carefully measure the chromatographic retention indices. Figures 2-5 show examples of mass spectra obtained during sequential scans by the spectrometer of specific peaks eluting from the GC. The scan sequence number and nominal KI are shown with each spectrum (The nominal KI was used in the figures because, when the mass spectrometer scanned across a peak eluting from the GC, not all mixtures were separated into individual isomers; therefore the mass spectra from the leading edge of the peak may be different than those from the trailing edge).

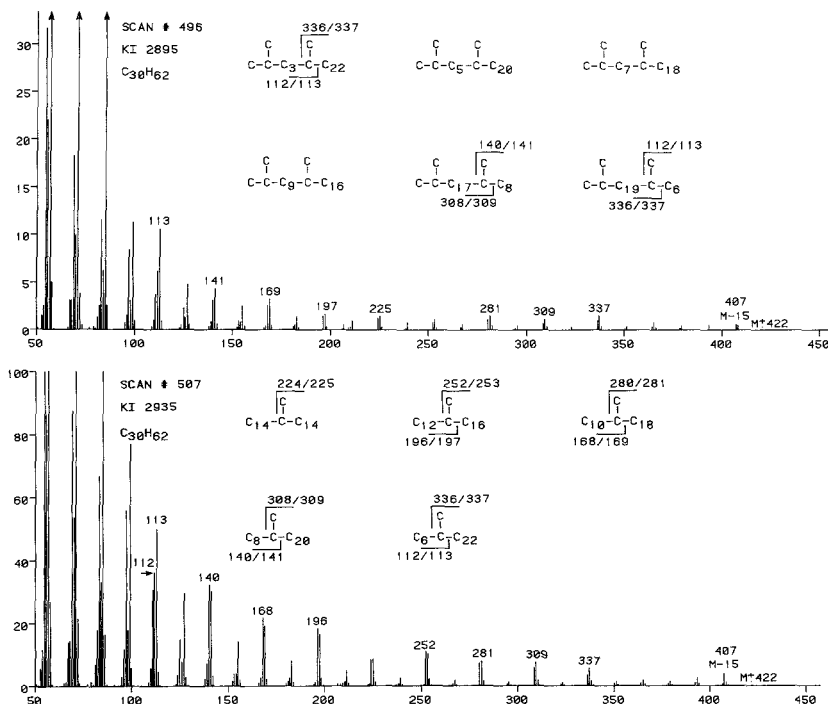


FIG. 2. Mass spectra of methylalkanes from screwworm: 2,X-dimethyloctacosanes (nominal KI 2895); methylnonacosanes (KI 2935).

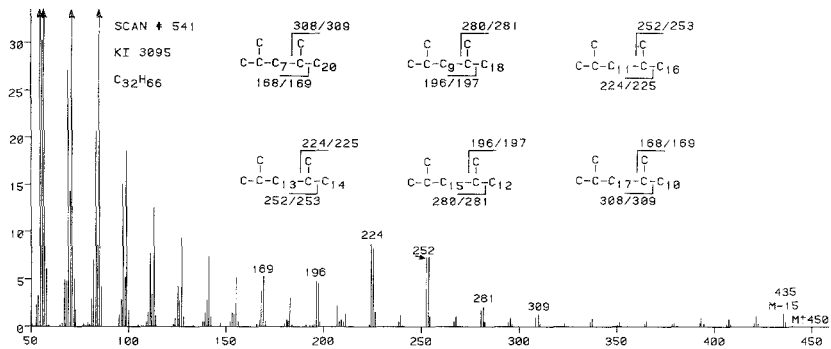


FIG. 3. Mass spectrum of mixture of 2,X-dimethyltriacontanes (nominal KI 3095).

The spectra shown in Figure 2 are from peaks eluting at nominal KI 2895 and 2935 and represent mixtures of 2,X-dimethyloctacosanes and methylnonacosanes. The two mixtures are isomeric to each other, have identical molecular weights, and identical mass spectra. They differ only in their retention indices. An additional series of 2,X-dimethyltriacontanes (nominal KI 3095) was identified from the hydrocarbon fraction (Figure 3). The 2,X-dimethylalkane structures were proven by synthesis and careful measurement of the KI values of homologous compounds (Pomonis et al., 1989).

Figure 4 shows the MS scan for the peak with nominal KI 3005. This spectrum for the mixture of 3,X-dimethylnonacosanes is typical of dimethylalkanes. The mass spectrum of the synthetic 3,11-dimethylnonacosane (Pomonis et al., 1980; Pomonis and Hakk, 1984; Rosenblum et al., 1976; Nishida et al., 1974) (KI 3006; Pomonis et al., 1989) showed that fragmentation external to carbon 11 yielded two fragments at m/z 182/183 with an intensity ratio > 1 ,

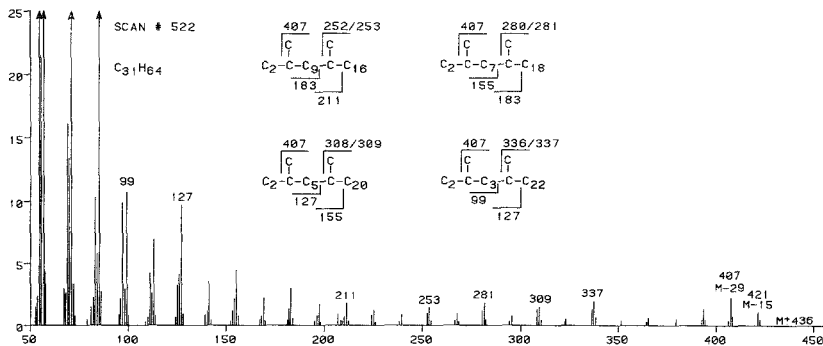


FIG. 4. Mass spectrum of screwworm 3,X-dimethylnonacosanes (nominal KI 3006).

TABLE 3. ALKENES OF SURFACE LIPIDS EXTRACTED FROM ADULT SCREWORMS

7-Octadecene	5-Tritriacontene
9-Octadecene	7-Tritriacontene
	9-Tritriacontene
7-Docosene	
7-Nonacosene	7-Pentatriacontene
9-Nonacosene	9-Pentatriacontene
5-Hentriacontene	7-Heptatriacontene
7-Hentriacontene	9-Heptatriacontene
9-Hentriacontene	

which suggests that the methyl branch at carbon 3 has no influence on fragmentation ratios, similar to the 2,X-dimethylalkanes. However, the mass spectrum of the mixture (Figure 4), which contains naturally occurring 3,11-dimethylnonacosane, does not show significant even mass peaks for those fragments from external scission of the internal methyl branch for all compounds, e.g., at m/z 210/211, 182/183, 154/155, 126/127. It is assumed that because there is a mixture, other fragments at these same m/z values may confound the expected ratios. Also, it should be pointed out that the mass spectrum (Figure 4) has been amplified 4 \times , suggesting that the compounds are present in very small quantities (Table 2). The strongest evidence for the 3,X-dimethylnonacosanes is the KI obtained for this peak (Pomonis et al., 1989). No other 3,X-dimethylalkanes were identified.

The alkene fraction had the largest variability among the various treatments and also the smallest quantity of mass of the hydrocarbons (Table 1). The amount of total alkenes remained fairly constant as the insects aged. The alkenes that were characterized by GC-MS as methoxy derivatives are listed in Table 3. Infrared spectrometry of the alkene mixture gave no peak at 970 cm^{-1} , which suggested *Z* rather than *E* double bonds (Jackson and Bartelt, 1986). Those alkenes identified were straight-chain monoalkenes with double bonds in position 5 or 7 or 9 (Table 3). No attempt was made to quantify the individual alkenes.

DISCUSSION

The insect's cuticular lipids may serve to protect it from desiccation, penetration of microorganisms and pesticides, and often may have a role in chemical communication (Jackson and Blomquist, 1976; Nelson, 1978; Blomquist and Jackson, 1979; Howard and Blomquist, 1982; Blomquist and Dillwith,

1985). Studies to develop understanding and knowledge of these processes may result in ecologically safe methods of insect pest control.

In an earlier gas chromatography study of the screwworm, Louloudes et al. (1962) compared the hydrocarbon fractions of extracts of whole body sodium hydroxide digests of several dipteran species. Even-numbered alkanes accounted for over one third of the total compounds in the screwworm. While all four species had alkenes, the screwworm had only 6% of the total hydrocarbons as unsaturates. The quantities of alkenes we found present in the screwworm were nearly identical with those reported Louloudes et al. (1962). Those authors reported that the principal constituents of the alkanes from the hydrocarbons of the screwworm were the C_{29} as well as appreciable amounts of C_{30} and higher homologs (about 25% of the material).

We found that the absolute amount of hydrocarbon increased in the screwworm female and male with age and may also be strain dependent (Pomonis and Mackley, 1985). Conversely, the hydrocarbon fractions contribute a decreasing percentage to the total quantity of surface lipid. At emergence, both sexes had nearly identical profiles and quantities of individual groups of compounds. Individual methylalkanes varied in amount with age for both sexes, suggesting sexual priorities in synthesis of certain compounds.

Comparison of irradiated and nonirradiated flies showed no difference between treatments as no large differences in quantities or quality of the various hydrocarbon groups were found. Apparently, synthesis and/or secretion is not affected by irradiation, possibly because the synthesis enzymes are already programmed before irradiation of the pupae. Irradiation of 1-day-old adult stable flies has been found to have no effect on hydrocarbons produced by these flies when measured at five days (Harris et al., 1976).

Normal, methyl- and dimethylalkanes, but not trimethylalkanes were found by GC-MS. All possible isomeric methylalkanes were found in the screwworm

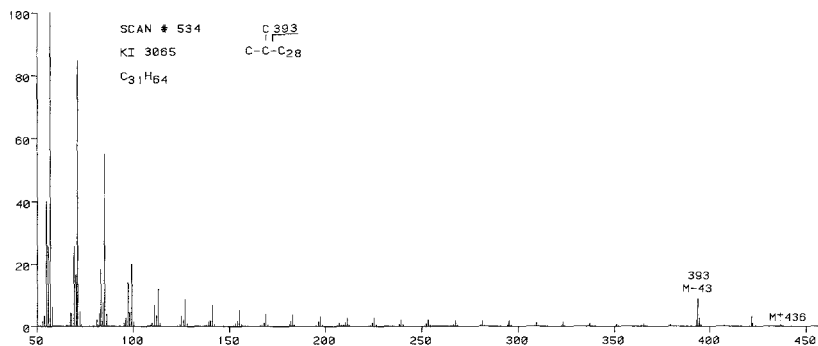


FIG. 5. Mass spectrum of 2-methyltriacontane (KI 3065).

except for 4-methylalkanes; however, not every isomer was found in every homologous series. In odd-numbered carbon backbone methylalkanes, the isomers were those in which the substitution was on odd-numbered carbons, e.g., 9-, 11-, and 13-methylpentacosane. In even-numbered carbon backbone methylalkanes, the isomers were those substituted on consecutive carbons e.g., 8-, 9-, 10-, 11-, . . . methyloctacosane.

Dimethylalkanes of several types were characterized from the screwworm surface hydrocarbon fraction. The ubiquitous dimethylalkanes with three methylene units between the branches (see Blomquist et al., 1987, for a recent review) were found also in the screwworm. The 3,X-dimethylalkanes have been previously reported in several species of insects (Nelson et al., 1980, 1981; Thompson et al., 1981; Lockey, 1982, 1984, 1985; Peschke and Metzler, 1987). In the screwworm, 3,X-dimethylnonacosanes (500 ng/fly) were detected only in the newly emerged female.

A group of novel compounds that eluted at KIs approximately equal to those of *n*-alkanes and whose spectra appeared to be those of monomethylalkanes in the next higher series were identified as belonging to a previously unreported class of dimethylalkanes, viz., 2,X-dimethylalkanes. Chromatographically, peaks KI 2895 and KI 3095 have KI values inconsistent for monomethylalkanes of their molecular mass, although their mass spectra appeared to be those of monomethylalkanes. Figure 2 compares mass spectra of the 2,X-dimethyloctacosanes and methylnonacosanes. These two groups of compounds give chromatographic peaks having a difference of about 40 units of KI. Identification was aided by synthesis and mass spectrometry of a series of 2,X-dimethylheptacosanes and careful measurement of the KIs (Pomonis et al., 1989). This group of compounds does not give the typical mass spectral fragmentation of dimethylalkanes. In previously reported spectra of dimethylalkanes (Nelson and Sukkestad, 1970; Nelson et al., 1972; Pomonis et al., 1978, 1980), characteristic fragments are found that are a result of scission about the methyl branch. This process yields an odd/even pair of ions of 1 amu difference for each methyl branch point. Additional characteristic fragment ions contain both methyl groups. However, the 2,X-dimethylalkanes do not give the typical spectra of the more frequently encountered internally branched dimethylalkanes. The methyl substituent on position 2 (the "iso" position) does not have an influence on fragmentation, so that the compound acts as if it has only one methyl group; that is, there is no significant peak indicating a loss of an isopropyl radical (very low intensity M-43, C₃H₇) so that the spectrum appears to be that of a monomethylalkane.

There was one ambiguity which was observed in the MS of these compounds: In Figure 2 there are groups of two compounds each, which may contribute the same fragments; for example, 2,10- and 2,18-dimethyloctacosane both yield ions at *m/z* 168/169 and 280/281 and 2,12- and 2,16-dimethylocta-

cosane yield ions at m/z 196/197 and 252/253, all of which result from fragmentation about the branch. Whether one or both isomers of each group are present in the natural products is not certain but must wait synthesis of examples of the 2,16- and 2,18-dimethylheptacosanes for comparison with the synthetic 2,10- and 2,12-dimethylheptacosanes (Pomonis et al., 1989).

This is the first report of the isolation and characterization of 2,X-dimethylalkanes from insects. The compounds may be present in other insect species and may have been overlooked because of their low concentration, their cochromatography with the *n*-alkanes that may not have been completely sieved out, and the similarity of their MS with the methylalkanes.

Current studies with carbon label suggests that quantitative differences exist in lipid content among different strains of screwworm. In contrast to the tsetse fly, where sexual dimorphism in the alkanes has also been shown and where mating activity has been found in the dimethylalkane fraction (Carlson et al., 1978), mating stimulant activity has not been found in the alkane or alkene fractions but is present in the more polar fractions of surface extracts. The results of these studies will be presented in future papers.

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SYNTHETIC METHYL- AND DIMETHYLALKANES Kovats Indices, [^{13}C]NMR and Mass Spectra of Some Methylpentacosanes and 2,X-Dimethylheptacosanes

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Abstract—All possible isomeric mono-methylpentacosanes as well as 2,6-, 2,8-, 2,10-, 2,12-, and 2,14-dimethylheptacosane were synthesized. The [^{13}C]NMR shifts and the Kovats indices were determined, and the relationship to separation of isomeric mixtures of insect cuticular waxes are discussed. When several homologous series of hydrocarbon isomeric mixtures are to be separated, it is not practical to attempt complete gas-chromatographic separation of all possible isomers. The mass spectra of the 2,X-dimethylheptacosanes are presented and discussed.

Key Words—Insect hydrocarbon, methylpentacosane, 2,X-dimethylalkane(s), [^{13}C]NMR, mass spectra, Kovats index.

INTRODUCTION

Insect hydrocarbons and their role in the growth, development, and behavior of insects have been widely studied and reviewed (Howard and Blomquist, 1982; Lockey, 1985, 1988; Blomquist and Dillwith, 1985; Blomquist et al., 1987). Characterization of hydrocarbon structure has been accomplished by mass spectrometry with some ambiguity because of the lack of standard compounds for comparison. Several papers have described the synthesis and mass spectra of some dimethylheptacosanes (Pomonis et al., 1980), methylpentatriacontanes, methylheptatriacontanes, a dimethylpentatriacontane, and a dimethylheptatriacontane (Pomonis et al., 1978) for the purpose of corroborating the structure of

these compounds, which had first been isolated from the tobacco hornworm (Nelson and Sukkestad, 1970; Nelson et al., 1972). The structures were assigned solely on the interpretation of the mass spectra of the natural products.

Many hydrocarbon mixtures that are isolated from insects are blends of not only homologs but also of isomers. Separation of the component compounds from these mixtures is usually accomplished by gas chromatography (GC). Structural characterization is performed by determining their Kovats indices (KI) (Kovats, 1965) and combining GC with mass spectrometry (GC-MS). Although homologs may easily be separated by GC, separation of isomers is not so readily accomplished. Capillary GC provides the best resolution of the mixtures. However, because the natural product mixtures contain compounds that range from 23 carbons ($C_{23}H_{48}$) to greater than 40 carbons ($C_{40}H_{82}$), the GC conditions require temperature programming over a wide range to obtain a separation. Theoretical as well as technical considerations do not allow complete GC separations, even by the best capillary chromatography, of all isomers in a series, e.g., not all isomeric methylalkanes can be separated from each other. The reasons for these limitations will be shown and discussed.

Recent biosynthetic studies using ^{13}C -labeled precursors showed incorporation of the label into insect hydrocarbons (Blomquist et al., 1980; Dwyer et al., 1981; Dillwith et al., 1982). Analysis of the isolated hydrocarbons by ^{13}C nuclear magnetic resonance spectrometry ($[^{13}C]NMR$) enabled investigators to identify the exact location of the incorporated label by nondestructive analytical means. The above authors used $[^{13}C]NMR$ data obtained from our synthetic compounds to assign structure to their biosynthetic hydrocarbons.

All possible 12 monomethylpentacosanes ($C_{26}H_{54}$) were synthesized. The GC Kovats index and $[^{13}C]NMR$ for each isomer was determined and will be discussed. The spectra obtained from a tandem mass spectrometer (MS-MS) of each of the monomethylpentacosanes were reported elsewhere (Cerny et al., 1986). In addition to the methylpentacosanes, five 2,X-dimethylheptacosanes were synthesized, viz., 2,6-, 2,8-, 2,10-, 2,12-, and 2,14-dimethylheptacosane. This class of dimethylalkanes was recently reported for the first time and was isolated from the screwworm (Pomonis, 1989). This report verifies the structure of these natural products by synthesis, chromatography, and spectrometry of some analogs.

METHODS AND MATERIALS

Gas Chromatography. The purity of the synthetic intermediates and final products was evaluated by GC. Analyses were performed with a Varian model 3700 flame ionization instrument using a 12- or a 25-m \times 0.20-mm-ID fused silica capillary column [Hewlett-Packard (H-P) cross-linked methyl silicone 19091102, 0.33 μ m film]. A Varian multipurpose type injector in the split mode

with a glass frit insert was used for all capillary chromatography. Sample introduction was by injection using a 100:1 split ratio. The GC was programmed from 150 to 320°C at a variable rate (usually at 4 or 2°/min) with a 2-min initial isothermal and an 8-min final isothermal hold. Helium was the carrier gas at a flow rate of 0.77 ml/min (20 cm/sec) with a head pressure of 12.5 psig. Chromatographic data was reported by a Hewlett-Packard model 3390A integrator interfaced to the GC. The KI (Kovats, 1965) of each methylpentacosane or artificial mixtures of the methylpentacosanes were determined by isothermal gas chromatography at 210°C and *n*-C₂₅H₅₂ and *n*-C₂₆H₅₄ were used as the reference points. The KI of the dimethylheptacosanes were determined with *n*-C₂₇H₅₆, *n*-C₂₈H₅₈, and *n*-C₂₉H₆₀ as the reference points at an isothermal oven setting of 225°C.

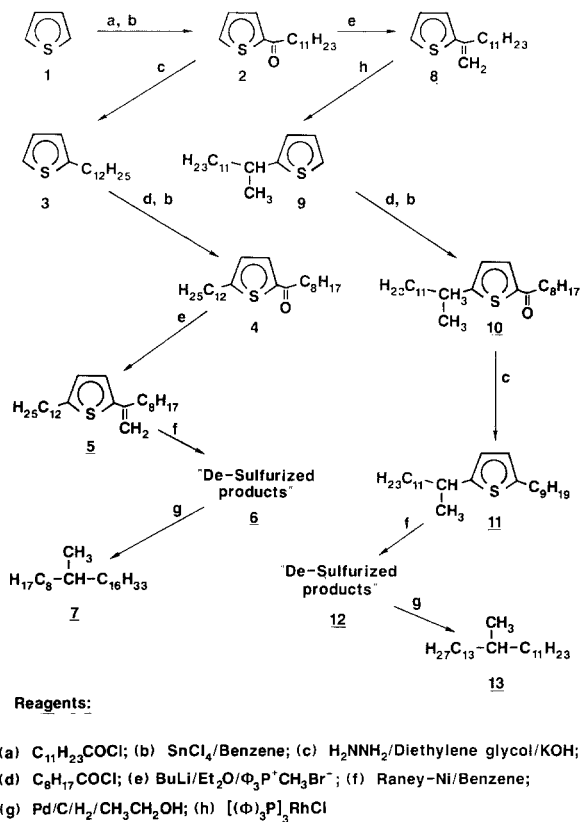
Gas Chromatography-Mass Spectrometry. These analyses were performed on a Hewlett-Packard model 5790 GC fitted with a 12.5-m methylsilicone fused silica capillary column with the exit inserted directly into the ionizing chamber of a Finnigan MAT model 1125 mass spectrometer. The transfer line from the gas chromatograph to the spectrometer was at 320°C. Ionizing voltage was 70 eV. The GC was programmed with a 2-min initial isothermal hold at 60°C, then heated to 150°C at 28°/min, then from 150 to 325°C at 3°/min using helium as carrier gas at 1 ml/min. An MS scan was made every 4.8 sec. Data acquisition and storage were made with a PDP 11/34 RSX-11 3.2 version computer dedicated to the spectrometer.

Synthesis. The procedures for synthesis of thiophene intermediates and methyl alkanes via Raney nickel desulfurization have been reported earlier (Pomonis et al., 1976a, b, 1978, 1980) and have not been modified significantly. Infrared and proton magnetic resonance spectra were in agreement with those previously reported (Pomonis et al., 1976a).

Carbon-13 Magnetic Resonance. The spectra were determined in CDCl₃ as 15% solutions with chemical shifts assigned relative to added tetramethylsilane (TMS) using a Jeol FX90Q magnetic resonance spectrometer at 22.5 MHz. Frequency assignments to carbons were calculated from the Lindeman and Adams (1971) formula shown in Levy and Nelson (1972).

RESULTS

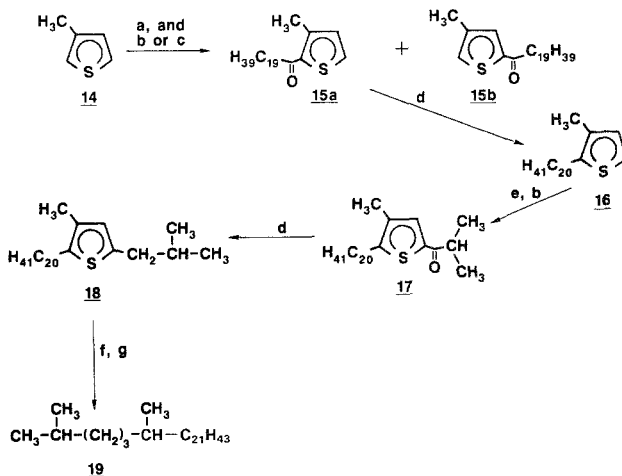
Synthesis. Synthesis of two of these methylpentacosanes is shown in Scheme 1. Friedel-Craft acylation of thiophene (**1**) with dodecanoyl chloride in benzene as solvent using stannic chloride as the Lewis acid gave 2-dodecanoylthiophene (**2**) in 82% yield. Modified Wolff-Kishner reduction of **2** gave the 2-dodecylthiophene (**3**) in 78% yield. Further acylation of **3** with nonanoyl chloride and stannic chloride gave **4** in near quantitative yield. Subsequent reaction of **4** with methyl Wittig reagent yielded the methylidene **5** (71% yield),



SCHEME 1.

which was desulfurized with Raney nickel and hydrogenated over palladium-carbon catalyst to give 9-methylpentacosane (**7**) in 61% yield from **5**. Compound **2** was also used to synthesize **13**. Methyl Wittig reagent, when reacted with **2**, gave **8** in 83% yield. To avoid desulfurization and/or poisoning of the heterogeneous catalyst during reduction of the methyldiene double bond, the homogeneous catalyst Tris-triphenylchlororhodium (Osborn et al., 1966; Harmon et al., 1969) was used to synthesize **9** from **8** (61%). Friedel-Crafts acylation, Wolff-Kischner reduction, desulfurization, and catalytic reduction (Pd/C) of **9** yielded **13** (**10**, 99%; **11**, 65%; **13**, 89%).

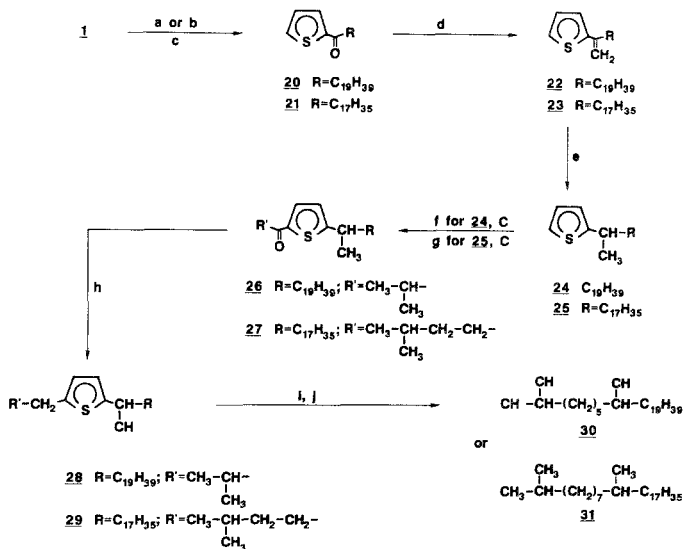
The 2,X-dimethylheptacosanes were synthesized as outlined in Schemes 2 and 3. Acylation of 3-methylthiophene (**14**) with eicosanoyl chloride gave a mixture of varying proportions of the two possible isomers: 2-acyl-3-methylthiophene (**15a**) and the 5-acyl-3-methylthiophene (**15b**). The ratio of 2-acyl to 5-acyl product was controlled by the type of Lewis acid used. Thus, when



Reagents:

(a) $\text{C}_{19}\text{H}_{39}\text{COCl}$; (b) $\text{SnCl}_4/\text{benzene}$; (c) $\text{AlCl}_3/\text{CS}_2$; (d) H_2NNH_2 , Diethylene glycol, KOH; (e) $\text{CH}_3-\text{CH}(\text{CH}_3)-\text{COCl}$; (f) Raney Ni; (g) $\text{Pd/C}/\text{H}_2/\text{CH}_3\text{CH}_2\text{OH}$

SCHEME 2.



Reagents:

(a) $\text{C}_{19}\text{H}_{39}\text{COCl}$; (b) $\text{C}_{17}\text{H}_{35}\text{COCl}$; (c) SnCl_4 , benzene; (d) BuLi , Et_2O , $\Phi_3\text{PCH}_2^+ \text{Br}^-$; (e) $(\Phi_3\text{P})_3\text{CIRh}$, H_2 , $\text{CH}_3\text{CH}_2\text{OH}$; benzene; (f) $\text{CH}_3-\text{CH}(\text{CH}_3)-\text{COCl}$; (g) $\text{CH}_3-\text{CH}(\text{CH}_3)-\text{CH}_2-\text{COCl}$; (h) H_2NNH_2 , diethylene glycol, KOH; (i) Raney-Ni/benzene; (j) Pd/C , H_2 , $\text{CH}_3\text{CH}_2\text{OH}$

SCHEME 3.

aluminum chloride was used, greater than 98:1 yield of **15a**:**15b** was obtained, while a ratio of 66:44 (**15a**:**15b**) was obtained when stannic chloride was employed. The two isomers were easily separated by alumina column chromatography. Compound **15a** was reduced to **16** by the Wolff-Kischner reaction (73%) and then acylated with 3-methylpropanoyl chloride and stannic chloride to give **17** (90%). Reduction of **17** to **18** (80%) followed by desulfurization and hydrogenation provided the 2,6-dimethylheptacosane (**19**, 90%). Thiophene (**1**) was acylated with either eicosanoyl or octadecanoyl chloride and stannic chloride to provide **20** (99%) or **21** (Scheme 3). Compound **20** was purified by column chromatography through alumina. These acylthiophenes were reacted with methyl Wittig reagent to provide either **22** (62%) or **23**, and the methylene double bond was subsequently reduced in a hydrogen atmosphere with homogeneous catalyst to prevent premature ring desulfurization to give **24** (92%) or **25**. Compounds **24** and **25** were acylated with 2-methylpropanoyl chloride and 4-methylpentanoyl chloride to give **26** (99%) and **27**, respectively, which were then reduced to give **28** (79%) and **29**. Raney nickel desulfurization of the dialkylthiophenes **28** and **29** followed by catalytic reduction with hydrogen over palladium on charcoal gave 2,8- and 2,10-dimethylheptacosane (**30** and

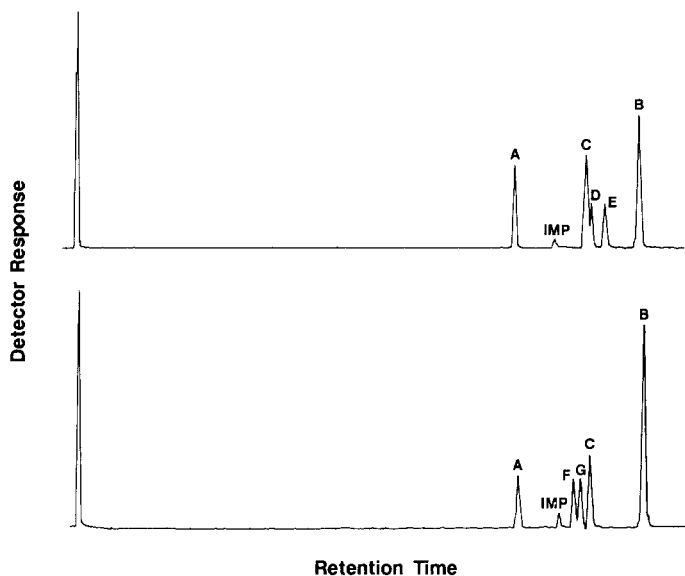


FIG. 1. Top: Capillary GC trace of A: $n\text{-C}_{25}\text{H}_{52}$ (KI 2500); B: $n\text{-C}_{26}\text{H}_{54}$ (KI 2600); C: 4-methylpentacosane (KI 2557); D: 2-methylpentacosane (KI 2562); E: 3-methylpentacosane (KI 2574). Isothermal at 210°C, 12.5-m methylsilicone fused silica column. Bottom: Capillary GC trace of A: $n\text{-C}_{25}\text{H}_{52}$; B: $n\text{-C}_{26}\text{H}_{54}$; C: 4-methylpentacosane; F: 6-methylpentacosane (KI 2544); G: 5-methylpentacosane (KI 2549). Isothermal at 210°C, 12.5-m methylsilicone fused silica column.

TABLE 1. KOVATS INDICES (KI) OF ALL POSSIBLE MONOMETHYLPENTACOSANES^a, SOME INTERNALLY BRANCHED DIMETHYLALKANES,^{b,c} AND SOME 2,X-DIMETHYLHEPTACOSANES^b

Compound	KI
3-Methylpentacosane	2574
2-Methylpentacosane	2562
4-Methylpentacosane	2557
5-Methylpentacosane	2549
6-Methylpentacosane	2544
7-Methylpentacosane	2541
8-Methylpentacosane	2538
9-Methylpentacosane	2536
10-Methylpentacosane	2534
11-Methylpentacosane	2533
12-Methylpentacosane	2532
13-Methylpentacosane	2532
9,11-Dimethylheptacosane	2773
8,14-Dimethylheptacosane	2771
9,14-Dimethylheptacosane	2768
9,13-Dimethylheptacosane	2765
2,6-Dimethylheptacosane	2808
2,8-Dimethylheptacosane	2802
2,10-Dimethylheptacosane	2802
2,12-Dimethylheptacosane	2794
2,14-Dimethylheptacosane	2794
3,11-Dimethylnonacosane	3006
10,14-Dimethyltriacontane	3061
13,17-Dimethylheptatriacontane	3752
15,19-Dimethylpentatriacontane	3551

^a Isothermal 210°C, 12.5-m methylsilicone (MeSi) fused silica capillary column.

^b Isothermal 225°C, 12.5-m MeSi fused silica capillary column.

^c Pomonis et al. (1980).

31, 88%) respectively. The 2,12- and 2,14-dimethylheptacosanes were similarly prepared.

Gas Chromatography. Each of the methylpentacosanes were gas chromatographed using various parameters on capillary columns to determine the best conditions for optimum resolution of isomers. The Trenzahl number (separation number) for these compounds was determined also using various chromatographic conditions. Chromatograms of mixtures of groups of three methylalkanes are shown in Figure 1. The best separation for this series was achieved on a 12-m × 0.22-mm-ID column with 1.0 or 0.33 μm film thickness at 210°C isothermal using helium as carrier gas at 30 cm/sec. The KI values of all methylalkanes that were determined in this study are listed in Table 1.

TABLE 2. CARBON-13 CHEMICAL SHIFTS (PPM) OF ALL POSSIBLE MONOMETHYLPENTACOSANES^a

	C ₁ ^b	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	C ₉
2-Methylpentacosane	22.62	31.98 ^c	39.10	27.98	29.35	29.74	29.74	29.74	29.74
3-Methylpentacosane	11.40	29.35	34.42 ^c	36.66	27.10	30.03	29.74	29.74	29.74
4-Methylpentacosane	14.43	20.18	39.49	32.57 ^c	37.15	27.10	30.13	29.74	29.74
5-Methylpentacosane	14.14	23.11	29.35	36.86	32.76 ^c	37.15	27.11	30.03	29.74
6-Methylpentacosane	14.14	22.82	32.86	26.91	37.25	32.74 ^c	37.25	27.21	30.13
7-Methylpentacosane	14.13	22.72	31.98	29.74	27.11	37.15	32.76 ^c	37.15	27.11
8-Methylpentacosane	14.14	22.82	32.08	29.55	30.13	27.21	37.25	32.86 ^c	37.25
9-Methylpentacosane	14.14	22.82	32.06	29.45	29.83	30.13	27.21	37.25	32.86 ^c
10-Methylpentacosane	14.15	22.73	31.99	29.46	29.75	29.75	30.04	27.12	37.16
11-Methylpentacosane	14.14	22.72	31.98	29.35	29.74	29.74	29.74	30.03	27.11
12-Methylpentacosane	14.06	22.74	31.91	29.38	29.77	29.77	29.77	29.77	30.06
13-Methylpentacosane	14.04	22.72	31.98	29.35	29.74	29.74	29.74	29.74	29.74

The KI values for the 2,X-dimethylheptacosanes show a trend toward decreasing values as the number of methylene groups between the methyl branches increases.

Carbon-13 Nuclear Magnetic Resonance Spectrometry (¹³C]NMR). The NMR spectra of all 12 methylpentacosanes as well as the five 2,X-dimethylheptacosanes were experimentally determined and the chemical shifts are listed in Tables 2 and 3, respectively. The tertiary carbon that bears the branched methyl group in the straight-chain portion has, as predicted by calculation (Levy and Nelson, 1972), a constant chemical shift for all possible isomers between 5- and 13-methylpentacosane. However, the tertiary carbons of 2-, 3-, or 4-methylpentacosane have chemical shifts that are significantly different from the more internally located tertiary carbons of the remaining isomers. This difference reflects the influence of the terminal methyl group at position 1. The branched methyl group, which is carbon-26 in all of the methylpentacosanes (Table 2), also has a fairly constant chemical shift in all possible isomers except for 2-methylpentacosane, in which the two ultimate methyl groups are equivalent. The chemical shift for carbon-26 (in 3-methylpentacosane also reflects the effect of the proximal carbons at positions 2 and 3.

Mass Spectrometry. The synthesis of the complete series of methylpentacosanes made compounds available for mass spectrometric fragmentation studies. These reactions were consistent with the well-known patterns of electron-impact-induced fragmentations for natural or synthetic methylalkanes (McCarthy et al. 1968; Pomonis et al., 1978, 1980). The spectra are not included in this paper.

TABLE 2. Continued

C ₁₀	C ₁₁	C ₁₂	C ₁₃	C ₁₄	C ₁₅	C ₁₆	C ₁₇₋₂₁	C ₂₂	C ₂₃	C ₂₄	C ₂₅	C ₂₆ ^d
29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.35	31.98	22.62	14.04	22.62
29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.35	31.88	22.72	14.04	19.20
29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.35	31.98	22.72	14.14	19.69
29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.35	31.98	22.72	14.14	19.69
29.83	29.83	29.83	29.83	29.83	29.83	29.83	29.83	29.44	32.08	22.82	14.14	19.79
30.03	29.74	29.74	29.74	29.74	29.74	29.74	29.74	29.35	31.98	22.72	14.13	19.70
27.21	30.13	29.84	29.84	29.84	29.84	29.84	29.84	29.55	32.08	22.82	14.14	19.79
37.25	27.21	30.13	29.83	29.83	29.83	29.83	29.83	29.45	32.06	22.82	14.14	19.79
32.77 ^c	37.16	27.12	30.04	29.75	29.75	29.75	29.75	29.46	31.99	22.73	14.15	19.70
37.15	32.76 ^c	37.15	27.11	30.03	29.74	29.74	29.74	29.35	31.98	22.72	14.14	19.70
27.13	37.08	32.79 ^c	37.08	27.13	30.06	29.77	29.77	29.38	31.91	22.74	14.06	19.72
30.03	27.11	37.15	32.76 ^c	37.15	27.11	30.03	29.74	29.35	31.98	22.72	14.04	19.70

^a 15% solutions in CDCl₃.

^b Carbon number of pentacosane backbone.

^c Denotes the carbon carrying the methyl substituent.

^d Carbon number of the methyl substituent.

The mass spectra of the 2,X-dimethylheptacosanes (Figures 2 and 3) reveal that a pair of peaks that are characteristic of fragmentation reactions typical of the more frequently encountered dimethylalkane compounds are missing. The spectra appear to be those of methylalkanes whose gas chromatographic KI values are about 40 units lower than those expected, i.e., very near the KI value of the *n*-alkane of one methylene unit less than the molecular ion mass of the experimental value.

DISCUSSION

Chromatography. In a companion paper (Pomonis, 1989) we described the isolation and characterization of a large number of hydrocarbons from the screwworm fly. We also attempted to quantify the individual hydrocarbons with some success. However, it is not always possible to separate an individual hydrocarbon component from all of its companion isomers even with capillary GC. Quantification of some individual hydrocarbons may therefore become difficult or impossible, causing ambiguity to be introduced; that is, only the mass of the mixture of hydrocarbons is represented by the chromatographic peak and measured.

The separation of two isomers on a GC column is a function of the col-

TABLE 3. CARBON-13 CHEMICAL SHIFTS (PPM) OF SOME SELECTED 2,X-DIMETHYLHEPTACOSANES^a

	1	2 ^b	3	4	5	6	7	8	9
2,6-dimethylheptacosane	23.01	28.27	39.69	25.15	37.63	33.06 ^c	37.44	27.40	30.32
2,8-dimethylheptacosane	22.72	27.99	39.10	27.11	30.33	27.50	37.15	32.76 ^c	37.15
2,10-dimethylheptacosane	22.62	27.98	39.10	27.11	30.03	29.74	30.03	27.11	37.15
	1	2	3	4	5	6	7-8	9	10
2,12-dimethylheptacosane	22.72	28.08	39.20	27.20	30.03	29.83	29.83	30.03	27.49
	1	2	3	4	5	6	7-10	11	12
2,14-dimethylheptacosane	22.62	27.98	39.10	27.10	30.05	29.93	29.93	30.05	27.40

umnn's separation number (n_{sep}) also known as the Trennzahl number, TZ (Ettre, 1977; Grob, 1977; Jennings, 1980). The TZ number is a measure of the possible number of resolved peaks between peaks of two members of a homologous series differing by one CH_2 unit. The TZ is inversely related to temperature, i.e., the higher separation numbers occur at lower temperatures. Thus, at lower temperatures, the peaks are separated by greater distances, but there is a point beyond which peak shape and resolution suffer. This implies that for efficient separation of moderate to higher molecular weight hydrocarbons at lower temperatures and large retention coefficients, inordinately long analysis times are required.

Our column, under optimized conditions, gave a $\text{TZ} = 34$ for separation of $n\text{-C}_{25}\text{H}_{52}$ and $n\text{-C}_{26}\text{H}_{54}$ according to the equation shown (Jennings, 1980):

$$\text{TZ} = [t_{R(n+1)} - t_{R(n)} / w_{0.5n} + w_{0.5n+1}] - 1. \quad (1)$$

It is possible to calculate the separation number necessary to separate two compounds whose KIs are known and which fall between the two n -paraffins (Jennings, 1980). The TZ is related to KI by the equation:

$$\text{TZ} = [100 / (\text{KI}_2 - \text{KI}_1)] - 1 \quad (2)$$

thus for 4-methylpentacosane (KI 2557) and 2-methylpentacosane (KI 2562) at 210°C isothermal on a 12.5-m capillary column, the relationship becomes:

$$\text{TZ} = [100 / (2562 - 2557)] - 1 = 19$$

Thus, the efficiency of the column has to be 19 or greater to resolve the two isomers. An example of this separation is shown in Figure 1 and includes a

TABLE 3. Continued

10	11	12	13	14-22	23	24	25	26	27	28 ^d	29 ^e
30.03	30.03	30.03	30.03	30.03	30.03	29.74	32.28	23.01	14.43	23.01	19.89
27.50	30.03	29.74	29.74	29.74	29.74	29.35	31.98	22.72	14.14	22.72	19.70
32.76 ^c	37.15	27.11	27.50	29.74	29.74	29.44	31.98	22.62	14.14	22.62	19.70
11	12	13	14	15	16-23	24	25	26	27	28 ^d	29 ^e
37.25	32.86 ^c	37.25	27.49	30.03	29.83	29.74	32.08	22.72	14.14	22.72	19.80
13	14	15	16	17	18-23	24	25	26	27	28 ^d	29 ^e
37.05	32.76 ^c	37.05	27.40	30.05	29.93	29.74	31.59	22.62	14.04	22.62	19.69

^a 15% solutions in CDCl₃.

^b Carbon bearing 1st methyl substituent (constant).

^c Carbon bearing 2nd methyl substituent (variable).

^d Carbon number of methyl substituent on carbon 2.

^e Carbon number of methyl substituent on variable carbon number.

third isomer, 3-methylpentacosane. Other factors that improve column efficiency are (1) increasing the linear flow velocity with use of hydrogen in preference to helium as carrier gas since hydrogen has a flatter van Deemter curve and (2) increasing the length of the column since resolution is a square root function of the column length (Jennings, 1980). However, increasing the column length to 50 m (4×12.5) only doubles the resolution ($R_s = 4^{1/2} = 2$). It also increases the analysis time and, under isothermal conditions, causes peak broadening. From equation 2 and from the KI values (Table 1) for various methylpentacosanes, it is seen that in order to separate some isomers a TZ = 99 (10-methyl- vs. 11-methylpentacosane) would be required. However, 10-methylpentacosane may more easily be separated from 4-methylpentacosane (TZ = 3.4).

From the foregoing argument, it is seen that the analyst who is dealing with a mixture of homologous hydrocarbons composed of several isomeric series such as those isolated from the insect surface lipids faces a formidable task if it is desired to separate the mixture into its individual component parts.

Magnetic Resonance Spectrometry. Isotope-labeled precursors such as malonic, 2-methylmalonic, propionic, and succinic as well as tetracosanoic acids have been used in studies to determine the metabolic origin of the methyl branches in insect hydrocarbons (Blomquist et al., 1980; Dwyer et al., 1981; Dillwith et al., 1982; Pomonis and Hakk, 1987). When radiolabel is biochemically incorporated in a molecule, the position of that label in the product is

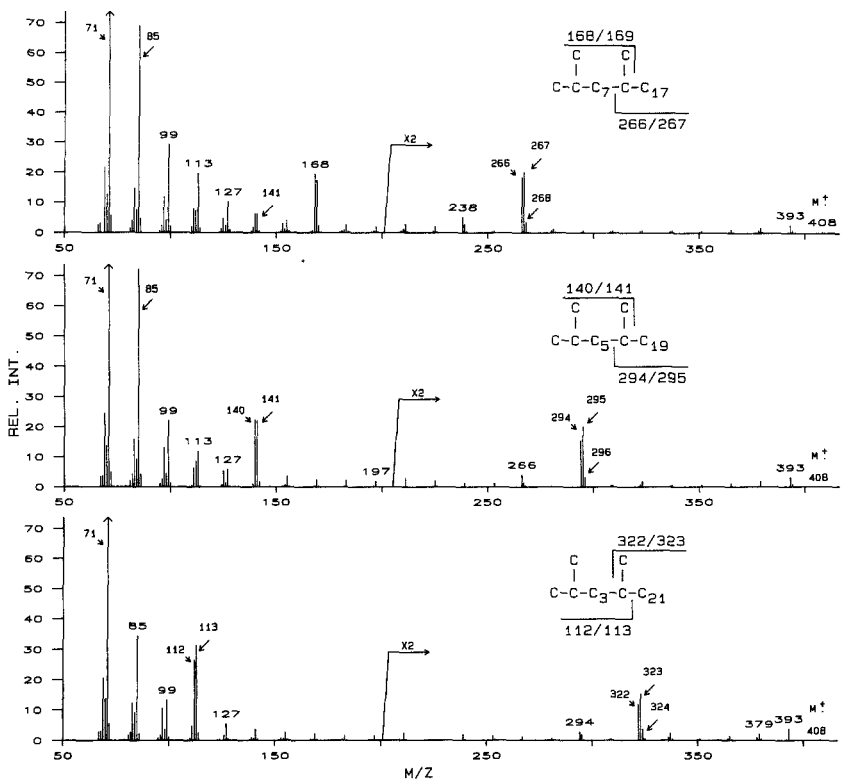


FIG. 2. EI mass spectra of synthetic compounds. Top: 2,10-dimethyl heptacosane (KI 2802). Middle: 2,8-dimethylheptacosane (KI 2802). Bottom: 2,6-dimethylheptacosane (KI 2808).

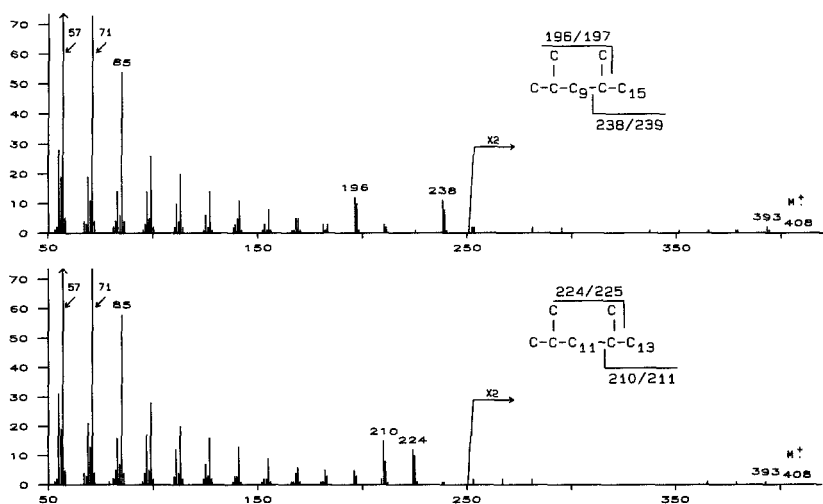


FIG. 3. EI mass spectra of synthetic compounds. Top: 2,12-dimethylheptacosane (KI 2794). Bottom: 2,14-dimethylheptacosane (KI 2794).

often determined by tedious and meticulous oxidative degradation. Alkanes are resistant to oxidative reactions, and the products of the oxidation are those of randomized cleavages leading to ambiguity as to the position of the label. However, ^{13}C -labeling allows the investigator to use nondestructive [^{13}C]NMR spectrometry to precisely observe the position of the incorporated label in the metabolic product. For example, [^{13}C]NMR analysis of the isolated hydrocarbon fractions from housefly surface lipids showed that the 2-methyl group in 2-[^{13}C]methylmalonic acid was the origin of the methyl branch in internally branched methylalkanes by isotope enrichment (Dillwith et al., 1982). The synthesis of all 12 methylpentacosanes permitted a systematic assignment of ^{13}C chemical shift values for each carbon in the molecules, with the experimental values (Table 2) being in very good agreement with the calculated values computed from an equation taken from Levy and Nelson (1972). A few of these experimentally determined chemical shift values were used in locating the position of the incorporated label (enrichment) in biosynthetic studies in the termite (Blomquist et al., 1980) and housefly (Dillwith et al., 1982).

Mass Spectra. In the companion paper (Pomonis, 1989) are described the isolation and characterization of a number of alkanes from the screwworm fly adult. Of special interest is the report of the characterization of 2,X- and 3,X-dimethylalkanes. The 2,X-dimethylalkanes are a new category of insect alkanes and were reported for the first time. Characterization of this group of alkanes was difficult because (1) they were present in very low concentration; (2) they were easily masked, chromatographically, under the *n*-alkane peak; and (3) most confounding of all, when all *n*-alkanes were removed by molecular sieving, two chromatographic peaks gave the same mass spectra. The unknown compounds were represented by a peak that chromatographed with a nominal KI of 2900 but presented mass spectra nearly identical to methylnonacosanes ($\text{C}_{30}\text{H}_{62}$), which are included in a peak with a nominal KI 2935 (Pomonis, 1989).

After considering several possible structures, the 2,X-dimethylalkane alternative seemed to fit the data but did not adhere to one of the several useful empirical rules of fragmentation for dimethylalkanes under electron impact (McCarthy et al., 1968; Nelson et al., 1972; Pomonis et al., 1980). Missing were those characteristically prominent single masses due to cleavage yielding fragment ions that include both methyl groups (Pomonis et al., 1980) and those due to loss of C_3H_7 (M-43). To test the 2,X-dimethylalkane hypothesis, several 2,X-dimethylheptacosanes were synthesized and the MS that were determined are shown in Figures 2 and 3. The fragmentation reactions of the synthetic compounds supported our earlier interpretations and assignment of structures as the 2,X-dimethylalkanes.

We have synthesized a number of 2,X-dimethylheptacosanes and determined and reported their mass spectra, [^{13}C]NMR, as well as their chromatographic retention indices (KI). These data corroborate the structures of a number of previously unreported compounds isolated from the screwworm fly (Pomonis,

1988). We also reported the synthesis, some spectral properties, and KI of the possible monomethylpentacosanes. The relationship of these physical properties to those of a few other methyl alkanes were compared and discussed.

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CHEMICAL MODEL FOR SHORT-TERM INDUCTION IN QUAKING ASPEN (*Populus tremuloides*) FOLIAGE AGAINST HERBIVORES

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Abstract—Simulated large aspen tortrix (*Choristoneura confictana*) herbivory of quaking aspen (*Populus tremuloides*) induces significant increases in concentrations of two phenol glycosides, salicortin and tremulacin, in leaves within 24 hr. Crushing of leaf tissue, as must occur when aspen leaves are eaten by chewing insects such as the large aspen tortrix, results in conversion of salicortin and tremulacin to 6-hydroxy-2-cyclohexenone (6-HCH). Salicortin, tremulacin, 6-HCH, and its degradation product, catechol, are all toxic to the large aspen tortrix when fed on an artificial diet. These damage-induced chemical changes provide a plausible mechanism for short-term resistance induced in aspen leaves by insect herbivory.

Key Words—Chemical induction, phenol glycosides, salicortin, tremulacin, 6-hydroxy-2-cyclohexenone, quaking aspen, *populus tremuloides* large aspen tortrix, *choristoneura confictana*, Lepidoptera, Tortricidae, chemical defense, short-term induction.

INTRODUCTION

Real or simulated insect herbivory can change the secondary chemistry of leaves and reduce their food value for insects within 24 hr (Rhoades, 1979, 1983; Haukioja, 1980; reviews in Raupp and Tallamy, 1989). These changes have

been attributed to a short-term induced defense (STID) response targeted by the plant at the attacking insect. The mechanisms of STID in some crop plants are well understood (e.g., Green and Ryan, 1972; Tallamy, 1985; Broadway et al., 1986) relative to the mechanisms of STID in woody plant leaves, which are poorly understood (Hartley and Lawton 1987; Jones and Coleman, 1989). Indeed, the existence of STID in woody plant leaves has been questioned (Fowler and Lawton, 1985; Myers, 1988).

In this paper, we report that simulated large aspen tortrix (*Choristoneura conflictana*) damage of quaking aspen (*Populus tremuloides*) leaves induces within 24 hr a sequence of changes in leaf phenolic glycoside chemistry that is potentially detrimental to the large aspen tortrix. We propose that these changes provide the first clearly defined chemical mechanism for STID in a woody plant.

Natural History of Quaking Aspen–Large Aspen Tortrix Interaction In Alaska. The natural history of the quaking aspen–large aspen tortrix interaction in Alaska has been described in detail elsewhere (Beckwith, 1968, and references therein). For the purposes of this study, suffice it to say that large aspen tortrix populations break out in interior Alaska at approximately 10- to 15-year intervals. During these outbreaks quaking aspen is severely (80–100%) defoliated early in the growing season (within three to four weeks of bud break) for two to four years in sequence by second to fifth instar larvae. When feeding upon aspen, larvae first spend several hours webbing themselves within two or more adjacent leaves (often all leaves on a single short shoot). Over the next two to four days the larvae eat these leaves. Thus, they feed upon damaged leaves for a sufficient period of time to experience the effects of the damage-induced changes we describe.

METHODS AND MATERIALS

Experimental Aspens. We studied 3-m-tall quaking aspen saplings growing on deep loess soils (Van Cleve and Oliver, 1982) near Fairbanks, Alaska. At the time of our experiments, these aspens had not been severely attacked by insects for 12 years, were experiencing minimal insect attack, and were located at least 5 km away from aspens experiencing significant insect attack. Thus, it is unlikely that either insect damage or communication between trees had altered their leaf chemistry (e.g., Rhoades, 1983) at the time we initiated experimental leaf damage.

In each experiment, several leaves on an aspen short shoot were damaged by tearing their edges to simulate damage caused by third to fifth instar tortrix larvae. Leaves from a similar short shoot on a different branch from the same

tree served as controls for this experiment. After 24 hr, damaged and control leaves were collected, immediately freeze-dried, and stored at -20°C until chemically analyzed (see below). Each individual aspen provided one replicate pair of leaves (control, damaged) for chemical analysis. This experimental design precluded pseudoreplication (Hurlbert, 1984), a problem in other studies of STID in woody species (Fowler and Lawton 1985). Because measurements of phenol glycoside levels were not normally distributed, a nonparametric test, the Wilcoxon paired-sample test (Zar, 1974) was used to test for significance.

We assayed leaves for phenol glycosides. To minimize the possibility of artifacts, fresh leaves were vacuum-dried immediately upon reaching the laboratory. Prior freezing or crushing of the leaves was not done because earlier observations (Lindroth and Pajutee, 1987; Clausen, unpublished) indicated that these procedures result in some conversion of salicortin and tremulacin into salicin and tremuloiden, respectively. Our methodology, however, did not result in these conversions because analyses of leaves collected late in the growing season indicated no salicin or tremuloiden unless leaves were frozen or crushed prior to drying (Clausen, unpublished). After drying, three leaves were soaked in 10.0 ml of absolute methanol containing 0.54 mg of pinosylvin as an internal standard. After soaking for 14 days at 10°C , the extracts were analyzed for phenol glycosides by HPLC using conditions reported earlier (Lindroth and Pajutee, 1987; Bryant et al., 1987).

Secondary Metabolite Bioassays. We incorporated aspen leaf phenol glycosides (3 and 6% dry wt) and their degradation products (1.6% dry wt; see below) into artificial diets (Bioserve; spruce budworm) supplemented with 1% freeze-dried aspen leaf powder as a feeding stimulant. In the cases of 6-hydroxycyclohexenone (6-HCH) and catechol, concentrations were used based on the salicortin/tremulacin diet (6% each) and calculations that assumed complete conversions (Figure 1). An unadulterated diet served as a control. Fifty larvae per diet were used in this experiment. Salicortin and tremulacin were isolated by the procedure of Lindroth et al. (1987). 6-HCH was prepared according to the procedure of Rubottom and Gruber (1978), and catechol was obtained from Sigma Chemical Co. Larvae were reared individually in 5-cm Petri dishes, and fresh food was added daily to ensure larvae never ran out of food. All larvae were reared at temperatures (24°C) and light conditions similar to that found in the crowns of quaking aspen early in the growing season when trees are attacked by second to fifth instar tortrix larvae.

Electrophilicity of 6-hydroxycyclohexenone (6-HCH). 2-Cyclohexen-1-one (40.7 mg, 0.42 mmol), 6-HCH (41.5 mg, 0.37 mmol), thiophenol (41.0 μl , 0.40 mmol), and camphor (38.7 mg, internal standard) were mixed in 1.0 ml of methanol and stirred at room temperature for 5 hr. GC analysis of the mixture was performed (RSL-200 column, 30 m \times 0.53 mm; flow rate of 6.0

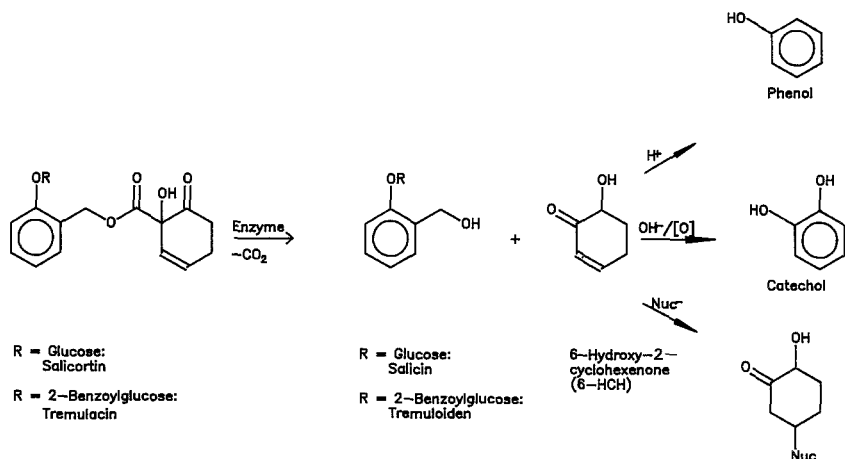


FIG. 1. Structures of aspen's major secondary compounds and their products following simulated herbivory.

ml/min He); temperature program of 80°C, 1 min; 80–150°C at 10°/min; 150°C, 2 min). The retention times for cyclohexenone, thiophenol, 6-HCH, and camphor were about 4.0, 4.4, 5.2, and 7.0 min, respectively.

The above experiment was repeated using similar amounts of reagents except that a hydrophobic solvent (benzene) was used and the reaction progressed for 48 hr prior to GC analyses.

Base-Catalyzed Oxidation of 6-HCH. A buffered (pH 10) aqueous solution (3 ml) of 6-HCH (ca. 50 mg) was stirred vigorously in an open flask. After 2 hr, the mixture was analyzed by TLC (silica gel; CH₂Cl₂/acetone, 95 : 5) using phenol, catechol, and 6-HCH as standards.

Enzyme Preparation. A crude enzyme preparation was obtained from aspen leaves by the procedure of Spencer and Seigler (1984). It was tested by adding salicortin (10 mg/ml) and, after incubation (pH 6.8, phosphate buffer; 24 hr; room temperature), analyzed by TLC (silica gel; CHCl₃/MeOH, 85 : 15) for the presence of salicortin/salicin and by GC-MS of an ether extract for the presence of 6-HCH. The above procedure was repeated using tremulacin as a substrate and analyzing for tremuloiden/6-HCH products as described above.

In Vivo Conversion of Tremulacin and Salicortin into Tremuloiden and Salicin, Respectively, and 6-HCH. Fresh leaves (containing salicortin and tremulacin but little salicin and tremuloiden) were cut along the main vein and divided into two samples. One sample was immediately extracted with methanol and served as a control. The other sample was ground in a mortar and pestle containing a water-sand mixture. After 30 min, the crushed sample was also extracted with methanol. After 24 hr of extraction, both samples were analyzed

by HPLC (for glycosides) and by GC (for 6-HCH). The experiment was repeated except, prior to crushing, leaves were heated to 70°C for 20 min to denature any leaf enzymes.

RESULTS

Quaking Aspen Leaf Secondary Chemistry. In agreement with the reports of Lindroth et al. (1987) and Pearl and Darling (1971b), we found four related phenol glycosides—salicin, tremuloiden, salicortin, and tremulacin (Figure 1)—in aspen leaves. Additionally, a volatile component, 6-hydroxy-2-cyclohexenone (6-HCH), was found in damaged leaf tissue. This substance has only been reported once before as a natural product (Mattes et al., 1987). Arguments will be presented in this paper that this substance is not an artifact of extraction (e.g., Lindroth and Pajutee, 1987) and, furthermore, that it is a component of STID in quaking aspen leaves.

Effects of Simulated Herbivory. Leaf damage, as occurs when second to fifth instar large aspen tortrix larvae feed on quaking aspen leaves, resulted in a significant ($P < 0.0025$, Wilcoxon paired-sample test) increase in leaf salicortin within 24 hr (Table 1). In addition, tremulacin ($P < 0.05$, Wilcoxon paired-sample test) levels increased following leaf damage only to a lesser extent

TABLE 1. LEVELS (mg/g LEAF) OF SALICORTIN AND TREMULACIN IN CONTROL (C) AND DAMAGED (D) LEAVES

Exp.	Date	Salicortin			Tremulacin		
		C	D	Increase	C	D	Increase
1	8/87	54.8	77.3	22.5	27.7	24.1	-3.6
2	8/87	86.1	104.4	18.3	46.9	48.8	1.9
3	8/87	49.2	65.2	16.0	27.6	30.3	2.7
4	8/87	106.9	114.9	8.0	61.7	63.4	1.4
5	8/87	113.4	109.0	-4.4	54.6	53.3	-1.3
6	8/87	96.8	97.1	0.3	47.7	42.5	-5.2
7	8/87	107.5	145.0	37.5	38.9	54.6	15.7
8	8/87	65.8	90.5	24.7	41.0	43.5	2.5
9	8/87	114.7	129.8	15.1	61.1	63.1	2.0
10	7/86	14.5	19.1	4.6	5.5	8.5	3.0
11	7/86	11.8	25.6	13.8	5.5	11.3	5.8
12	7/86	13.3	15.6	2.3	8.3	9.7	1.4
13	7/86	61.8	73.2	11.4	56.1	61.9	5.8
Total				13.1			2.5
Standard error				3.6			1.3

(Table 1). No such change was observed in the concentrations of either salicin or tremuloiden. Thus quaking aspen is capable of selectively altering the concentrations of phenol glycosides in its leaves upon leaf damage.

Additional crushing of leaf tissue, as must occur when it is chewed by insects, resulted in complete conversion of salicortin and tremulacin into salicin and tremuloiden, respectively, with a significant (ca. fivefold; $P < 0.05$, paired t test) increase in leaf 6-HCH (Figure 2). This second change did not occur in previously heated leaves, indicating that it is enzymatically mediated. Further evidence for the enzymatic nature of these conversions was gathered by treating salicortin with an enzyme preparation obtained from quaking aspen leaves using the procedure of Spencer and Seigler (1984) and observing the formation of both salicin and 6-HCH at the expense of salicortin. Tremulacin, on the other hand, did not convert to tremuloiden and 6-HCH when treated with the same enzyme preparation. In this latter case, the lack of an observable reaction may have been due to tremulacin's insolubility in the reaction medium. In neither case did a boiled enzyme preparation result in any conversions.

Bioassays. Incorporation of salicortin, tremulacin, and their mixture in artificial diets at a 3% (dry wt) concentration leaves had no significant effect upon pupal weights. However, incorporation of salicortin, tremulacin, and their mixture at 6% (dry wt) levels reduced pupal weights in comparison to the control diet (Figure 3; $P < 0.01$, all comparisons). In addition, both 6-HCH and catechol, at concentrations potentially formed in the insect gut from salicortin and tremulacin, reduced pupal weights relative to control ($P < 0.01$, both comparisons). The possibility that the activity of 6-HCH in artificial diets was due only to the decomposition products (catechol or phenol) was tested by analyzing

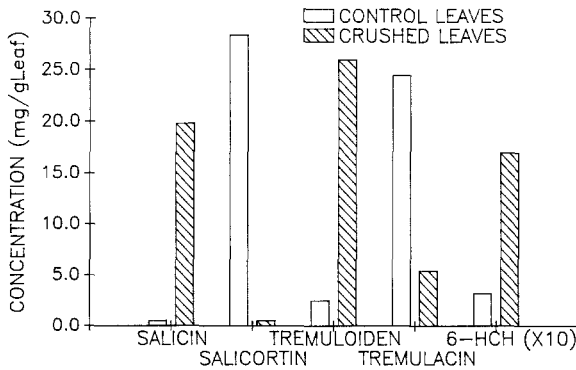


FIG. 2. Effect of leaf crushing on secondary metabolite levels. All changes are significant at $P < 0.001$.

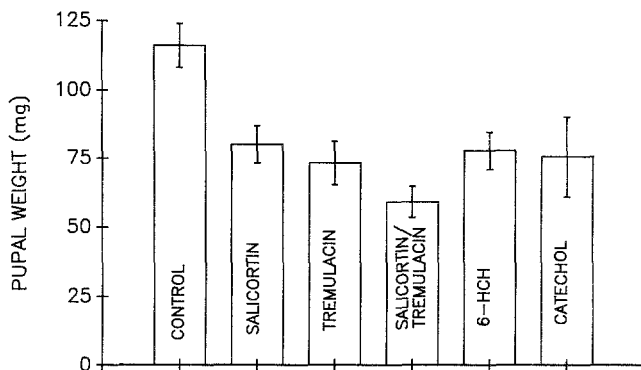


FIG. 3. Bioassay results on artificial diets. All comparisons with control diet are significant at $P < 0.01$. Concentrations of glycosides and their decomposition products were 6.0% and 1.6%, respectively.

unused 6-HCH diet for 6-HCH, catechol, and phenol by GC. Only about 30% of the original 6-HCH placed on the diet could be accounted for at the end of the feeding trial, but no phenol or catechol was detected.

Reactions of 6-HCH. When treated with base under aerobic conditions, 6-HCH was completely converted to catechol within 2 hr. In methanol, 6-HCH reacts with thiophenol slightly faster (about 10%) than 2-cyclohexen-1-one to give an addition product (Figure 4) whose structure was determined based on GC-MS and $[^1\text{H}]$ - and $[^{13}\text{C}]$ NMR spectra. Under hydrophobic conditions (benzene), however, 6-HCH reacted over twice as fast as 2-cyclohexen-1-one. Thus, especially in a hydrophobic environment, 6-HCH appears to be a potent electrophile, capable of reacting with biological nucleophiles (e.g., proteins, nucleic acids).

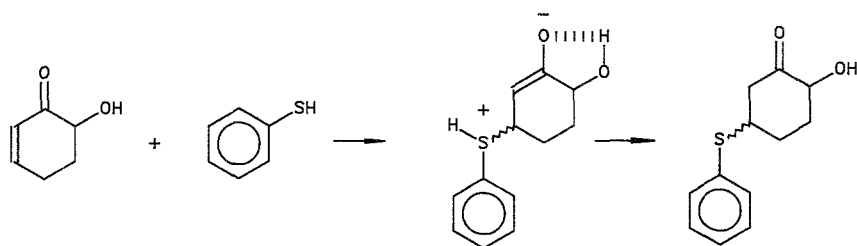


FIG. 4. Reaction mechanism of 6-HCH (an electrophile) with thiophenol (a typical nucleophile).

DISCUSSION

Herbivore-Related Consequences of Attacking Quaking Aspen Leaves. Mattson and Palmer (1987) found that simulated insect attack of quaking aspen leaves resulted in a significant increase in leaf phenols in damaged leaves. Our results indicate that this increase is a result of a rise in the concentrations of two of quaking aspen's four leaf phenol glycosides, salicortin and tremulacin. Although we have not systematically investigated the variables that could affect the magnitude of this response (e.g., nature and degree of leaf damage, induction time, seasonal effects, leaf age), our experimental procedure resulted in increases of ca. 13 and 3 mg phenol glycoside/g leaf tissue for salicortin and tremulacin, respectively. These increases potentially provide significantly enhanced defenses of leaves that originally had low levels of phenol glycosides but only marginal increases in the defense of previously well-defended leaves.

Furthermore, we found that ingestion of these two phenol glycosides has deleterious effects on the growth of large aspen tortrix larvae. This result is in agreement with our previous studies of effects of quaking aspen leaf secondary metabolites upon large aspen tortrix larvae (Bryant et al., 1987) and results of studies of quaking aspen leaf phenol glycosides on swallowtail butterfly larvae (Lindroth et al., 1988).

The increases in phenol glycoside content in aspen foliage following simulated herbivory can have significant effects on insect herbivores such as the large aspen tortrix and the swallowtail in the field. A large aspen tortrix larva confines itself within a group of leaves prior to feeding (Beckwith, 1968) and subsequently feeds for two to four days before emerging. Hence, the tortrix cannot avoid any induced changes that it has caused. A swallowtail larva, on the other hand, feeds on a single leaf for only a few hours before it leaves the leaf in search for a new one (MacLean, personal communication). This observation suggests that during feeding by the swallowtail, the food quality of the aspen leaf has declined sufficiently to eventually be rejected.

Crushing, as occurs when aspen leaves are eaten by chewing insects such as the large aspen tortrix or swallowtail butterfly, resulted in enzymatic conversion of salicortin to 6-HCH, a potentially very toxic natural product, with salicin as a by-product. Although we have not been able to obtain an enzyme preparation that converts tremulacin into 6-HCH and tremuloiden (presumably due, at least in part, to solubility restrictions), circumstantial experimental evidence (leaf crushing causes rises in 6-HCH and tremuloiden concentrations and a decrease in tremulacin levels) indicates that such an enzymatic conversion is likely. In addition, it is difficult to envision a nonenzymatic route that would allow these transformations under mild (room temperature, pH \approx 7) conditions. These results can also explain Lindroth and Pajutee's (1987) observation that certain extraction procedures cause aspen's leaf chemistry to change.

Irrespective of the relative contributions of salicortin and tremulacin to 6-HCH production, we observed a fivefold increase in 6-HCH concentration in crushed leaf tissue. This increase is a conservative estimate of the actual rise in 6-HCH in crushed leaf tissue because of the reactivity of this substance with leaf tissue (if 6-HCH is painted on leaves, they turn black immediately; Bryant, personal observation). However, our results (Figures 2 and 3) indicate that 6-HCH, at concentrations that can be formed from moderate levels of salicortin and tremulacin is sufficient to reduce the food quality of aspen leaves as food for second to fifth instar large aspen tortrix larvae.

Such a reduction in quality can be expected for several reasons. Under basic conditions, as occur in the gut of many forest defoliators, 6-HCH will rapidly convert to catechol (this paper; Pearl and Darling, 1971a), a substance that reduces the growth of tortrix larvae (e.g., Figure 3) and is toxic to other herbivores (Reese and Beck, 1976; Todd et al., 1971). Although less likely [because of the basic/oxidative conditions in most Lepidoptera guts (Isman, personal communication)], conversion of 6-HCH to phenol (a toxic substance; Sax, 1979) upon exposure to mildly acidic conditions (Mattes et al., 1987) provides another potentially detrimental effect of ingestion of 6-HCH. Additionally, 6-HCH, somewhat similar in structure to 4-hydroxy-2-cyclopenten-1-one (a substance toxic to herbivores because of its electrophilic properties; Spencer, 1987; Hall et al., 1977), can act as an electrophile.

Source of Salicortin and Tremulacin. There are two mechanisms that could cause the damage-induced rise in salicortin and tremulacin we observed in aspen leaves: (1) de novo synthesis from recent photosynthate and/or (2) translocation of salicortin and tremulacin from internodes or nearby leaves upon insect attack. While we cannot completely rule out the first possibility, circumstantial evidence supports the translocation mechanism. Current growth internodes of the quaking aspen population we studied contain salicortin and tremulacin in high concentrations (about 10 and 6%, respectively) and no salicin or tremuloiden (Clausen et al., 1989). Thus, translocation of phenolic glycosides from internodes to leaves could account for the rapid increase in salicortin and tremulacin and absence of an increase in the concentrations of salicin and tremuloiden that we observed in damaged aspen leaves. Experiments of MacLeod and Pridham (1966) provide further evidence for such a mechanism, and Tallamy (1985) has demonstrated that translocation of cucurbitacins is a likely mechanism of short-term induced defense (STID) in squash.

Proposed Mechanism for Short-Term Induced Defense in Quaking Aspen Leaves. We conclude by proposing a clearly defined, easily testable chemical model for STID in quaking aspen leaves (Figure 5). Upon initial attack (biting), the levels of salicortin and tremulacin rise in the damaged leaf within 24 hr. This rise is largely a result of upon-demand translocation of salicortin and tremulacin from internode stores to leaves, although some de novo synthesis by

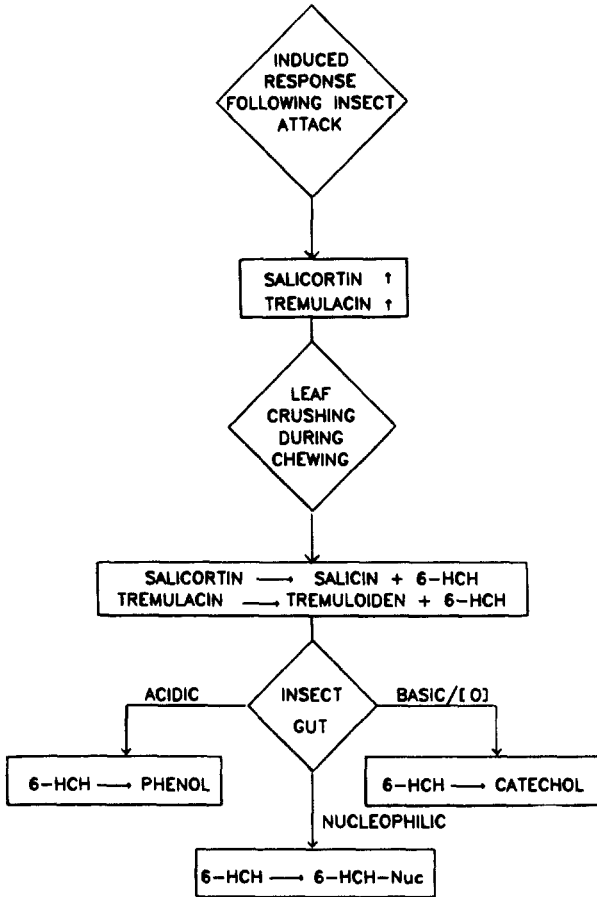


FIG. 5. Chemical model for short-term induction in quaking aspen.

the damaged leaves or leaves “plumbed” to the damaged leaves cannot be precluded as a contributing factor. Upon ingestion, salicortin and tremulacin are converted to 6-HCH by aspen leaf esterases. Within the herbivore’s gut, 6-HCH is converted to one of several toxins, such as catechol or phenol, depending upon gut conditions, or is trapped by nucleophiles. These chemical changes reduce the food value of aspen leaves for defoliators (such as the tortrix) that feed upon the damaged leaves for 24–72 hr. Validation of this mechanism for STID in quaking aspen would provide strong support for the Haukioja-Rhoades short-term induced defense hypothesis (Rhoades, 1979; Haukioja, 1980).

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INDUCTION OF CYTOCHROME P450-MEDIATED DETOXIFICATION OF XANTHOTOXIN IN THE BLACK SWALLOWTAIL

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Abstract—Xanthotoxin is a phototoxic allomone found in many of the host plants of the black swallowtail, *Papilio polyxenes* (Lepidoptera: Papilionidae). When added to the diet of final instar larvae, xanthotoxin can induce the cytochrome P450 monooxygenase (P450) activity in midgut microsomes by which it is detoxified. Induction is dose-dependent, increasing sevenfold when larvae feed on parsley treated topically with xanthotoxin at 0.5 or 1.0% fresh weight. Although xanthotoxin exerts much of its toxic effects when photoactivated by ultraviolet light, induction of P450 activity did not differ in the presence or absence of ultraviolet light. Despite a 4.7-fold induction of xanthotoxin-metabolizing P450 activity, total P450 content measured in the same microsomal samples did not increase significantly. These data indicate that multiple forms of P450 exist in the black swallowtail midgut and that they are differentially induced by xanthotoxin.

Key Words—Black swallowtail, *Papilio polyxenes*, Lepidoptera, Papilionidae, microsomal cytochrome P450, induction, xanthotoxin, furanocoumarins, plant defensive compounds.

INTRODUCTION

Cytochrome P450 monooxygenases (P450s) are heme proteins that function in the oxidation of a wide range of endogenous and xenobiotic substrates. Because plants produce a diversity of secondary metabolites that act as defensive compounds, many potentially toxic xenobiotics are encountered by organisms that

feed on plants. Studies on selected insects, notably lepidopterous larvae, have shown that many plant secondary metabolites act as inducers of P450 (e.g., Brattsten et al., 1977; Brattsten, 1987; Yu, 1987). However, as discussed by Dowd et al. (1983), Gould (1984), and Nitao (1989), P450 activity following treatment with plant allomones has, in almost all cases, been assayed against model substrates such as insecticides rather than against the inducing substrates themselves. Thus, the nature of the interaction between P450s and plant allomones has been examined for only a few natural systems. In this study, we examined ecological and physiological factors affecting P450 metabolism of a natural substrate, xanthotoxin, a furanocoumarin, by the black swallowtail, *Papilio polyxenes* (Lepidoptera: Papilionidae), which commonly encounters furanocoumarins in its host plants.

Furanocoumarins are secondary metabolites characteristic of the plant families Umbelliferae (=Apiaceae) and Rutaceae. The black swallowtail feeds exclusively on plants in these two families, and several of its hosts contain an abundance of xanthotoxin (Berenbaum and Feeny, 1981). Ivie et al. (1983) determined that tolerance of black swallowtail larvae to xanthotoxin is conveyed by rapid detoxification in midgut tissue and identified two oxidized metabolites of xanthotoxin in the excreta of larvae. It was subsequently determined that detoxification occurs principally in the microsomal fraction of the midgut, is NADPH-dependent, and is inhibited by piperonyl butoxide (Bull et al., 1986), suggesting that xanthotoxin metabolism in the black swallowtail is mediated by P450. Nitao (1989) found that the parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Oecophoridae), a specialist on two umbellifers high in xanthotoxin content, also detoxifies xanthotoxin by P450 activity. Moreover, he demonstrated that xanthotoxin induces the P450 activity by which it is detoxified.

We investigated whether midgut P450 detoxification of xanthotoxin can be similarly induced in the black swallowtail, and we examined several additional aspects of xanthotoxin detoxification in this species, namely pH optimum, dose dependence, effect of ultraviolet (UV) light, and increase in total P450 content. Dose-dependent induction of P450 detoxification of xanthotoxin in the black swallowtail is of potential ecological significance in that the content of this allomone varies among host species (Berenbaum, 1981), within host populations (Berenbaum et al., 1986), and temporally and anatomically within a single host (Nitao and Zangerl, 1987; Zangerl and Bazzaz, 1989). Xanthotoxin and many other furanocoumarins exert at least part of their toxic activity, i.e., cross-linking of DNA strands and generation of oxygen radicals, upon photoactivation by UV light (Murray et al., 1982). However, it is not known whether photoactivation of xanthotoxin has an effect on its induction of cytochrome P450. Finally, there is strong evidence that insects, like mammals (Nebert and Gonzalez, 1987), have multiple forms of P450 (Hodgson, 1985). We compared the increase of P450-mediated xanthotoxin metabolism with the increase in

overall P450 content to determine whether a subset of P450 isozymes is induced, thus providing insight into the degree of specialization of P450 in oligophagous insect herbivores.

METHODS AND MATERIALS

Chemicals. Xanthotoxin was purchased from Sigma Chemical Co., St. Louis, Missouri, and from Aldrich Chemical Co., Milwaukee, Wisconsin. All other reagents, unless otherwise noted, were purchased from Sigma.

Insects. A laboratory colony of the black swallowtail was established with females collected in east central Illinois in June 1988. Larvae were maintained at 21°C and 70% relative humidity, on parsley, *Petroselinum sativum* (Italian flat leaf), grown in a plot on the University of Illinois at Urbana-Champaign campus. Parsley was chosen as host plant because of its low xanthotoxin content. The xanthotoxin content of leaves sampled from our plot in June and August ranged from 0.003 to 0.009% of fresh weight (fw), as determined by a method slightly modified from Berenbaum et al. (1984).

For all experiments, newly molted final instar larvae were transferred from untreated parsley to fresh parsley sprigs treated topically with either acetone or an acetone solution of xanthotoxin. Xanthotoxin solutions of different concentration were prepared, such that equal volumes (0.25 ml/g fw parsley) could be applied to obtain diets containing topically added xanthotoxin at 0.1, 0.5, and 1.0% fw. Larvae were reared on the treated parsley for three days before microsomes were isolated.

For experiments testing the effect of UV light on P450 induction by xanthotoxin, larvae were reared under 40-W BLB bulbs (General Electric Co., Cleveland, Ohio). Cages containing control larvae were shielded with Plexiglas UV screens opaque to wavelengths of less than 400 nm. Using a model UVX Digital Radiometer (Ultra-Violet Products, San Gabriel, California), the intensity of UVA light (approximately 320–420 nm) was measured as 270 $\mu\text{W}/\text{cm}^2$ in treatment cages, and as 5 $\mu\text{W}/\text{cm}^2$ in control cages.

Assays of P450 Activity and Content. Midguts were dissected from larvae and placed in sucrose medium [0.25 M sucrose, 1% polyvinylpyrrolidone, 1.1 mM EDTA (pH 7.5), and 2 mM phenylmethyl sulfonyl fluoride (PMSF)], then slit open, cleared of food, and rinsed in fresh medium (Crankshaw et al., 1979; Nitao, 1989); these and all further manipulations were carried out on ice. The midguts were ground in 3–10 volumes of sucrose medium (as described, except containing 0.5 mM PMSF and 5 $\mu\text{g}/\text{ml}$ leupeptin, a broad-spectrum protease inhibitor) by hand with 10 strokes of a ground glass tissue homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000g for 10 min at 4°C. The 10,000-g supernatant was centrifuged at

100,000g for 1 hr at 4°C, and the resulting microsomal pellet was resuspended in cold 0.1 M sodium phosphate (pH 7.8) containing 0.5 mM PMSF and 5 µg/ml leupeptin.

Assays measuring xanthotoxin-metabolic activities were run for 30 min at 30°C in a shaking water bath. One-milliliter reactions contained 0.1 M sodium phosphate (pH 7.8), 0.3 mM NADP, 3.0 mM glucose-6-phosphate, 0.5 units glucose-6-phosphate dehydrogenase, 10 µg xanthotoxin, and 0.05–0.2 mg protein. Reactions were started with the addition of microsomes and stopped with 250 µl 2 N HCl. After acidification, 10 µg of bergapten, an isomer of xanthotoxin, were added to each assay for use as an internal standard. Reaction mixtures were extracted twice with one volume of ethyl acetate, and the organic phases were evaporated to dryness. The samples were resuspended in 100 µl ethyl acetate and the amounts of xanthotoxin and bergapten were measured by high-pressure liquid chromatography, slightly modified from Berenbaum et al. (1984).

For all experiments, time zero control assays (equal in number to the number of duplicates run per treatment) were prepared, acidified before the addition of microsomal protein but otherwise treated identically to reaction flasks. To compensate for any substrate loss not attributable to enzymatic activity, the mean amount of substrate quantified in time zero controls was assumed to be the amount of substrate present in each reaction at the start of the enzyme assay. The amount of xanthotoxin metabolized in each reaction was calculated by subtracting the amount of xanthotoxin remaining in each flask at the end of the reaction from the mean amount of substrate at time zero.

Since the activity of P450 is affected by the pH of the assay conditions (Hodgson, 1985), we conducted a series of assays in reaction mixtures ranging from pH 7.0 to 9.0. The microsomes used were from final instar larvae reared on parsley treated with xanthotoxin at 0.1% fw. We observed a broad optimum for xanthotoxin metabolism ranging from pH 7.5 to 8.0 (Table 1) and conducted all subsequent experiments at pH 7.8. We also verified that the metabolism of xanthotoxin in our system was linear with respect to protein concentration and the 30-min incubation period.

Bull et al. (1986) demonstrated that xanthotoxin metabolism by black swallowtail midgut microsomes is carried out by mixed function oxidases, the activity of which is dependent on the presence of NADPH. To determine if increased metabolism due to prior exposure to xanthotoxin is also mediated by NADPH-dependent enzymes, we conducted preliminary xanthotoxin metabolism assays in the presence and absence of NADPH-generating systems. In two experiments with microsomes from induced larvae, xanthotoxin metabolism in assays lacking NADPH averaged only 6% of that in assays containing the NADPH-generating system.

Total P450 content was determined by carbon monoxide difference spectra

TABLE 1. EFFECT OF pH ON *in Vitro* P450-MEDIATED METABOLISM OF XANTHOTOXIN BY MIDGUT MICROSOMES OF BLACK SWALLOWTAIL LARVAE

pH	Xanthotoxin metabolized (nmol/min/mg protein) ^a
7.0	4.60 ± 0.52a
7.5	5.43 ± 0.09b
8.0	5.61 ± 0.51b
8.5	4.51 ± 0.09a
9.0	4.20 ± 0.39a

^aMeans ± SD of one experiment assayed in quadruplicate. One-way ANOVA showed a significant effect of pH on xanthotoxin metabolized ($F_{4,15} = 10.76$, $P = 0.003$). Means followed by the same letter are not significantly different at $P = 0.05$, pairwise comparisons using LSD.

(Omura and Sato, 1964). For each experiment, one or two samples from each microsomal protein preparation were analyzed, and two scans were run on each sample. Determinations of protein concentration in samples were made with protein dye from Bio-Rad Chemical Division, Richmond, California, using bovine serum albumin as a standard. Data were analyzed by one-way or two-way ANOVA with the following main effects included in the analyses: pH (Table 1), presence of UV light (Table 2), and xanthotoxin added to diet (Tables 2, 3, and 4).

RESULTS AND DISCUSSION

Xanthotoxin metabolism in the midgut of the black swallowtail is inducible, increasing approximately threefold and sevenfold when larvae are fed parsley treated with xanthotoxin at 0.1% and 0.5% or 1.0% fw, respectively (Table 2). The induction process appears to be dose-dependent, but maximum induction is reached at or before a diet with a xanthotoxin content of 0.5% fw. This result may reflect physiological adaptation to the levels of xanthotoxin encountered by the black swallowtail in its diet in nature. Of its three most common hosts in eastern North America, wild parsnip, *Pastinaca sativa*, wild carrot, *Daucus carota*, and poison hemlock, *Conium maculatum* (Feeny et al., 1985), xanthotoxin content is highest in wild parsnip, ranging up to 1% dry weight (dw) in foliage and ripe fruits (Zangerl and Berenbaum, personal observations). Since the parsley foliage used in our study was 83% water by fw, our xanthotoxin levels of 0.1, 0.5, and 1.0% fw correspond to approximately 0.59, 2.9, and 5.9% dw, respectively.

TABLE 2. EFFECT OF DIETARY XANTHOTOXIN CONTENT ON INDUCTION OF P450-MEDIATED METABOLISM OF XANTHOTOXIN IN MIDGUT MICROSOMES OF BLACK SWALLOWTAIL LARVAE

Diet (% fw xanthotoxin)	Xanthotoxin metabolized (nmol/min/mg protein) ^a
0	1.10 ± 0.30a
0.1	3.01 ± 0.39b
0.5	8.33 ± 0.76c
1.0	7.92 ± 1.73c

^aMean ± SD of two experiments, assayed in triplicate. One-way ANOVA showed a significant effect of diet on xanthotoxin metabolized ($F_{3,20} = 81.11$, $P = 0.0001$). Means followed by the same letter are not significantly different at $P = 0.05$, pairwise comparisons using LSD.

There are at least two ways in which a black swallowtail larva is likely to encounter differing xanthotoxin content in its diet within a single instar and, therefore, benefit from an inducible xanthotoxin-metabolic P450 activity. First, these caterpillars feed on the flowers, seeds, and foliage of umbelliferous plants (Scott, 1986), and all of these parts are often available to a larva during a single instar. The content of xanthotoxin and other furanocoumarins in the reproductive parts of the wild parsnip increases as a reproductive unit matures through bud, male flower, female flower, and ripe fruit stages (Nitao and Zangerl, 1987). Second, furanocoumarin content in parsnip leaves can be induced by UV light (Zangerl and Berenbaum, 1987), mechanical damage, and insect feeding (Zangerl and Bazzaz, 1989).

The amount of induction of P450 metabolism of xanthotoxin did not differ between larvae reared in the presence or absence of UVA light (Table 3); this result, however, may be due to the use of UV light of an intensity insufficient to photoactivate xanthotoxin to the degree necessary to effect the induction process. The intensity of UVA light measured on a sunny day in east central Illinois, 1500–1900 $\mu\text{W}/\text{cm}^2$, appreciably exceeds the value of 270 $\mu\text{W}/\text{cm}^2$ used in this study. However, in experiments using UV lighting comparable to ours, the southern armyworm, *Spodoptera eridania* (Lepidoptera: Noctuidae) (Berenbaum, 1978), and the corn earworm, *Heliothis zea* (Lepidoptera: Noctuidae) (Berenbaum, 1985), both polyphagous herbivores, suffered higher mortality when fed xanthotoxin in the presence of UV light, compared to control larvae shielded from UV light. Thus, xanthotoxin is apparently photoactivated under these lighting conditions. We conclude, therefore, that photoactivation of xanthotoxin is not necessary to initiate the induction process in the black swallowtail.

TABLE 3. EFFECT OF ULTRAVIOLET LIGHT ON INDUCTION OF P450-MEDIATED METABOLISM OF XANTHOTOXIN BY MIDGUT MICROSOMES OF BLACK SWALLOWTAIL LARVAE

UV light	Diet (% fw xanthotoxin)	Xanthotoxin metabolized (nmol/min/mg protein) ^a
No	0	1.51 ± 0.67a
No	0.1	4.35 ± 0.52b
Yes	0	1.52 ± 0.44a
Yes	0.1	4.05 ± 0.34b

^aMean ± SD of two experiments, assayed in triplicate. Two-way ANOVA showed no significant effect of UV light on xanthotoxin metabolized ($F_{1,19} = 1.75$, $P = 0.20$), and no significant UV light*diet interaction ($F_{1,19} = 0.00$, $P = 1.00$). Effect of diet on xanthotoxin was significant ($F_{1,19} = 164.85$, $P = 0.0001$). Means followed by the same letter are not significantly different at $P = 0.05$, pairwise comparisons using LSD.

Finally, despite a 4.7-fold induction in specific activity against xanthotoxin, total P450 content did not increase significantly (Table 4). These data indicate that multiple forms of P450 exist in the black swallowtail midgut and that a select subset of these is induced by xanthotoxin. If there were only a single form of P450 in the insect, then, assuming induction proceeding by de novo synthesis of enzymes (Hodgson, 1985) and a constant rate of substrate turnover, a fivefold induction of activity against xanthotoxin would require an equivalent increase in total P450 content.

Prior exposure to plant secondary compounds may not only induce P450-mediated metabolism of some substrates but may, at the same time, decrease activity against others (Yu, 1984; Brattsten, 1987). Yu (1984) found a 2.6-fold

TABLE 4. INDUCTION BY XANTHOTOXIN OF TOTAL P450 CONTENT AND OF P450-MEDIATED METABOLISM OF XANTHOTOXIN BY MIDGUT MICROSOMES OF BLACK SWALLOWTAIL LARVAE

Diet (% fw xanthotoxin)	Total P450 content (nmol/mg protein) ^a	Xanthotoxin metabolized (nmol/min/mg protein) ^a
0	0.159 ± 0.036a	2.02 ± 0.81a
0.5	0.180 ± 0.035a	9.44 ± 2.84b

^aMean ± SD of 2 experiments. P450 content assayed with one or two duplicates per treatment and two scans per duplicate. Enzyme activity assayed in triplicate. Means within a column sharing the same letter are not significantly different at $P = 0.05$, pairwise comparisons using LSD.

induction of total P450 content when fall armyworm larvae were reared on artificial diet containing 0.025% fw xanthotoxin. However, P450 activity against three of four model substrates decreased, while heptachlor epoxidase activity was induced 1.9-fold. It may be that, at a dietary content of 0.5% fw, xanthotoxin also decreases the level of some P450s in the black swallowtail, explaining in part why we observed no significant increase in total P450 content.

The presence of multiple forms of P450, with different and often overlapping substrate specificities, makes the suite of these enzymes a general and flexible detoxification system. However, it is clear that this system can be altered by selection and that it differs in composition among herbivores with different diets. For example, as discussed by Nitao (1989), the parsnip webworm, restricted in North America to two host species high in xanthotoxin content, has an uninduced *in vitro* capacity of xanthotoxin metabolism 30 times greater than that of the black swallowtail (Bull et al., 1986; this study), which feeds on several genera in two plant families, and 200 times greater than that of the fall armyworm (Bull et al., 1986; Yu, 1987) and cabbage looper, *Trichoplusia ni* (Lee and Berenbaum, unpublished), generalists reported from over 15 plant families (Tietz, 1972). In addition, xanthotoxin can induce the P450 activity by which it is detoxified in the parsnip webworm (Nitao, 1989) and black swallowtail (this study), but not in the cabbage looper (Lee and Berenbaum, 1989). The nature of this specialization of P450 monooxygenase systems probably lies in the evolution of particular isozymes and of pathways involved in the induction process for greater activity against defensive compounds characteristic of the diets of different herbivores.

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PATTERN OF PHEROMONE-ORIENTED FLIGHT IN MALE POTATO TUBERWORM MOTHS

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Abstract—The pheromone-oriented flight of male *P. operculella* was observed under field conditions. Eighty percent or more of the flights were shorter than 1 m. Thus the approach of males of this species to a distant pheromone source is composed of a chain of short flights. The pattern of the flights changed when the males came within about 1 m of the lure, with a decrease of flight speed and an increased tortuosity. This probably facilitates eventual location of the female by a male. Only a few males succeeded in arriving at the high-dose lures. Males did not succeed in arriving at the lure when it was 70 cm above the ground: this was confirmed by a trap test; however, the flight pattern consisted of a chain of short flights even in these cases. The adaptive significance of this flight pattern is discussed.

Key Words—Lepidoptera, Gelechiidae, *Phthorimaea operculella*, oriented flight, sex pheromone, flight pattern, field behavior.

INTRODUCTION

There are many studies on the behavior serving mate location by male moths. In some examples the behavior is divided into two stages; flight from a relatively distant site and a change of behavior close to the female, often involving landing and walking (e.g., Cardé, 1979). There are few records of the flying approach in the field, mainly because of difficulty in following the flying moths. David et al. (1983) have analyzed flight patterns of male *Lymantria dispar* approaching a distant odor source in the field. Kawasaki (1981) has observed flight behavior of male *Spodoptera litura* in a field cage. Both studies showed that the approach to a distant odor source in these two species was achieved by continuous flight.

On the other hand, a number of studies have dealt with close-range behavior. Some of them showed hovering searching (zigzag flight) near the female (or lure) after a flying approach (Cardé, 1981; Hidaka, 1972), or landing and searching by walking, sometimes while wing-fanning (Baker et al., 1976; Cardé et al., 1975).

In contrast, the oriented movement towards a distant odor source in male potato tuberworm moths, *Phthorimaea operculella*, appears to be composed of a chain of short flights (Ono, 1985).

The present observations were, therefore, designed to analyze this behavior quantitatively and to compare the effects of different lures and of increased pheromone concentration on the upwind oriented flight of this species.

METHODS AND MATERIALS

The observations were conducted with males (2–3 days old) reared in a laboratory culture on potatoes (25°C, 14-hr light–10-hr dark regime) and then released in the field. This culture had been kept since 1983 when the larvae and pupae were collected from a potato field in Nagoya. A large cloth sheet (5 × 17 m) with a numbered grid of 10-cm squares (8500 squares total) was placed on the ground without crops, and males' behavior during approach to a lure (a rubber septum containing pheromone) on the sheet was observed. The rubber septum, on a stick 10 or 70 cm high, was set at an upwind position, and males were released from downwind. Males responding to the lure were followed until they arrived at the source, in the case of successful orientation, or until they flew away from the sheet or spent more than 5 min without successful arrival. The behavior and landing site (grid square number) were recorded on cassette tapes which were later transcribed. Marks were put on the transcribed track at 5-sec intervals.

Ideally, the behavior of the nocturnal moths should be observed under natural darkness. Such observations of *P. operculella* are impossible because the moths are very small and their movements are rapid. The observations were, therefore, conducted around sunset (about 1800 hr) between July and September 1986. Lights-off in the rearing room was set at 1600 hr, and males were kept separately from females in the room under the dark conditions (25°C) until just before testing. This procedure did not seem to alter their behavior.

To randomize the influence of daily conditions, all lures should ideally be examined on the same day. On the other hand, this procedure could cause contamination of the sheet with the previously tested lures. In these experiments, we examined one sample a day and attempted to minimize the differences of daily weather conditions by avoiding rainy or windy days.

All the analyses were done from the trials in which it was possible to trace

flight tracks longer than 4 m on the sheet. Flight length was measured from the transcribed tracks (Figure 1). Flight length was defined as the beeline length between the center points of grid squares where males landed subsequently. Also, "flight speed," "advance speed," and "tortuosity" were defined as shown in Figure 2. Flight length (Df) was defined as the summed length of the transcribed track, and flight speed (ground speed) was calculated as Df/time (sec) elapsed. Stop durations longer than 5 sec in each were excluded from the "time" used in this calculation. Advance speed was calculated from beeline length as Db/time (sec) elapsed. Tortuosity was defined as the $Df1/Db1$ ratio from the start to the point of the first crossing of the 2-m line, and $Df2/Db2$ ratio from that point of first crossing to the lure or the end point of observation. This analysis is similar to that of Nakamuta (1985).

Trapping tests were conducted in the same field. Two water-pan pheromone traps (30 cm in diameter and 15 cm in depth) containing water with detergent were placed 5 m apart. One of the traps was placed on a stand and the other directly on the ground. The lures were set at 70 cm and 10 cm above the ground, respectively. One hundred twenty males were released 10 m downwind from the traps, and the number of males caught in each trap was counted on the following day. The observations were repeated eight times.

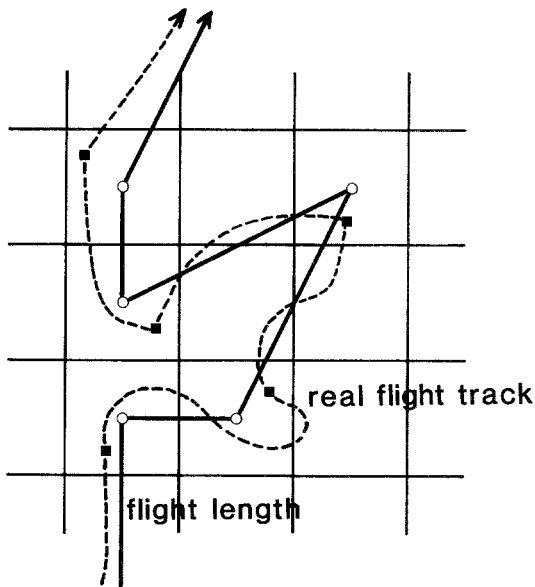


FIG. 1. Schematic method for measuring flight length. Flight length was defined as the beeline length between the center points of grid squares where males landed subsequently. ■: landing point and ○: center point.

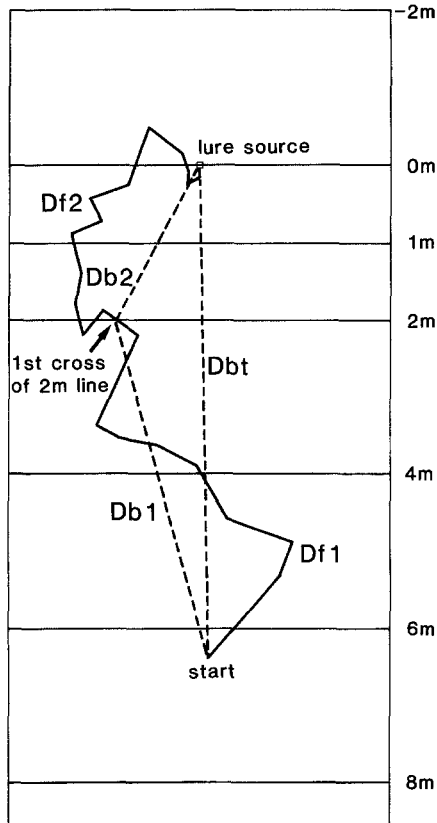


FIG. 2. Parameters for calculation of flight speed, advance speed, and tortuosity. Df1 and Df2: flight length of start to 2-m line and that of 2-m line to lure (end). Db1, Db2, and Dbt: beeline length of start to 2-m line, 2-m line to lure (end), and start to lure (end), respectively.

Female crude extract (FE: 100 female equivalents) and synthesized pheromone components, (*E,Z,Z*)-4,7,10-tridecatrienyyl acetate (T) and (*E,Z*)-4,7-tridecadienyyl acetate (D), were used as lures. Six treatments were tested: female extract 10 cm and 70 cm high (FE 10 and FE 70), T 30 μg + D 20 μg at 10 cm and 70 cm (TD 10 and TD 70), T 50 μg at 10 cm (T 10), and T 1200 μg + D 300 μg at 10 cm (HIGH 10). The data for FE 70 are excluded from this report because only two trials produced results.

TABLE 1. PERCENTAGES OF MALES ARRIVING AT LURE AND STOPPING ON THE WAY

Treatment		No. of males observed	Males arriving at lure (%)	Males stopping (> 5 sec) during their approach (%)	No. stops/trial
FE	10	10	90	60	1.6
TD	10	11	100	30	0.3
T	10	17	100	18	0.3
HIGH	10	13	38	31	0.4
TD	70	9	0	67	2.1

RESULTS

Table 1 shows the percentages of males arriving at the lure and showing a brief "stop" on the way. Arrival percentages at lures set at 10 cm were very high except for HIGH 10 (significantly different from other 10-cm data, 5% in Fisher's exact probability test). Although males approached close to the HIGH 10 lure (range: 30–200 cm), many of them (8/13) suddenly flew away. No males arrived at the source with TD 70.

FE 10 induced significantly more stops (> 5 sec) than TD 10 and T 10, and TD 70 significantly more than TD 10, T 10, and HIGH 10, at the 5% level in the Mann-Whitney U tests, respectively.

The frequency distribution of flight length and the percentages of flights longer than 1 m are shown in Figure 3 and Table 2. The longest flight recorded was 3.8 m. Table 2 shows that a majority of flights observed were shorter than 1 m with all the lures tested, although the percentages of flight longer than 1 m increased slightly as the distance from the lure increased. These facts support the conclusion that the approach flight of *P. operculella* males is not a continuous flight but a chain of short flights.

The lures in these tests were 10 cm above the ground (except TD 70). A possible factor inducing the short flight pattern noted in this experiment might be the low position of the lures (Figure 4). To check this point, observations were made with the lures at 70 cm above the ground. Males failed to arrive at these sources. In these experiments, males flew into the vicinity of the lures (five of nine approached within 200 cm of the source) but could not find the lure itself (Table 1). Their approach pattern differed from that in TD 10 (Figure 5), and they made frequent stops during the course of oriented flight. The distribution of flight lengths in the experiment was, however, similar to that when the lures were 10 cm high (Figure 3 and Table 2).

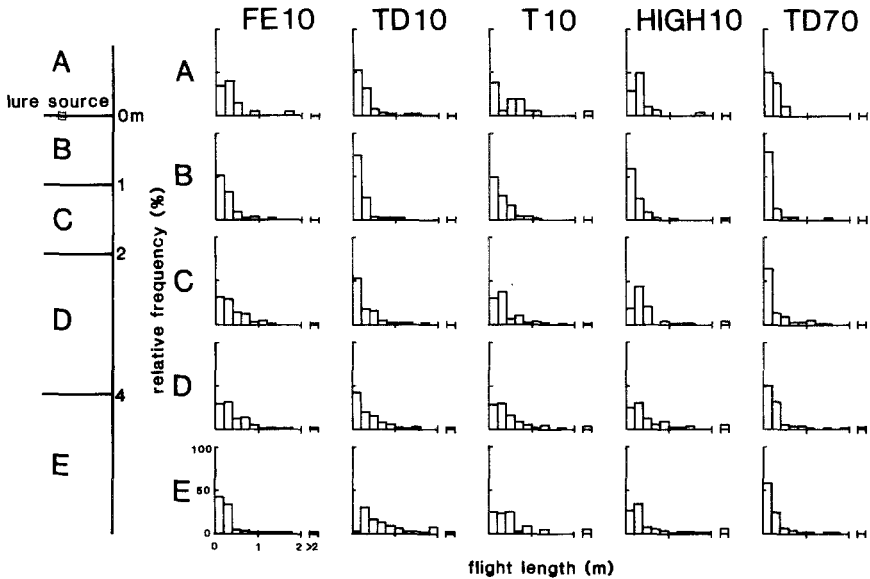


FIG. 3. Frequency distribution of flight length in each range of distance (A-E).

This fact was confirmed by a trap test. Many more males were caught at 10 cm than at 70 cm (Table 3). The short flight approach is, therefore, thought to be normal in *P. operculella*.

Table 4 shows the summarized data on the flight length in each range of distance. Flight lengths in the 1- to 0-m range were shorter than those in the >2-m range, except in the case of TD 70. In TD 10, flights in the 2- to 1- and 0- to -2-m range were also shorter than those >2 m.

TABLE 2. PERCENTAGES OF FLIGHTS LONGER THAN 1 m DURING ORIENTED FLIGHT OF POTATO TUBERWORM MOTH

Treatment	Percent of flights longer than 1 m (N)			
	-2 to 0 m	0 to 1 m	1 to 2 m	>2 m
FE 10	5 (20)	2 (144)	8 (80)	10 (182)
TD 10	2 (97)	1 (179)	5 (55)	16 (147)
T 10	13 (16)	1 (154)	10 (72)	13 (116)
HIGH 10	4 (28)	4 (97)	12 (43)	18 (203)
TD 70	0 (8)	3 (38)	7 (119)	4 (318)

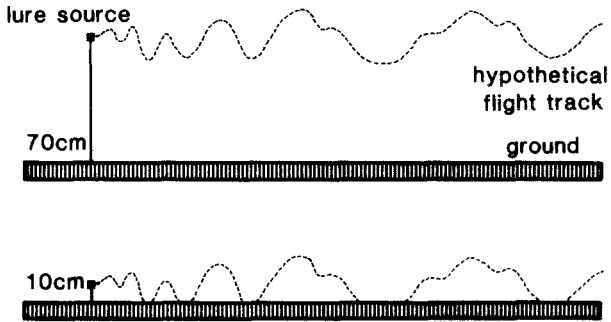


FIG. 4. A hypothetical flight track to the lure set at 70 and 10 cm high. It shows the possibility that the same flight path is convertible continuous flight and chain of short flights depend on lure height.

Flight speed and tortuosity of the track are shown in Table 5. The data were separated into two portions for each lure, as in Figure 2. No differences were observed among lures set at 10 cm, although the flight speed in TD 70 differs from others (Duncan's multiple-range test). Differences between start to

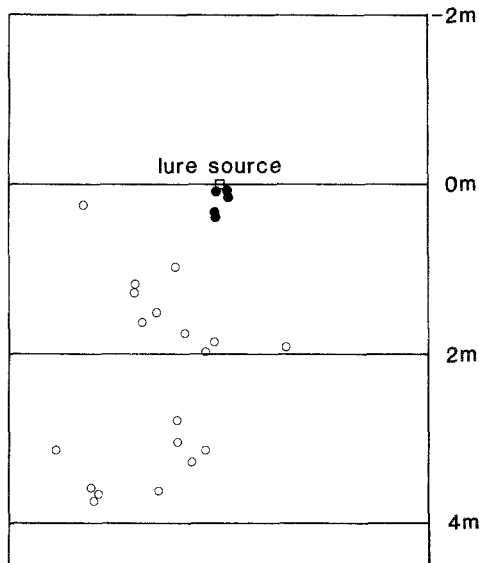


FIG. 5. Position showing stop (>5 sec) on the way to the TD 10 (●) and TD 70 lures (○). Number of males tested and number of stops observed were 11 and 5 in TD 10, and 9 and 19 in TD 70, respectively.

TABLE 3. EFFECT OF LURE HEIGHT ON TRAP CATCH

Height (cm)	No. males caught
70	142 ^a
10	536

^aTotal number out of eight replications in each of which 120 males were released. Catches significantly different at 1% (binomial test).

TABLE 4. COMPARISON OF FLIGHT LENGTH AMONG LURE TREATMENTS^a

Treatment	Mean length of a flight (cm)			
	>2 m	2 to 1 m	1 to 0 m	0 to -2 m
FE 10	44.5	44.1(ns)	25.3(1%)	35.9(ns)
TD 10	57.0	33.1(1%)	19.5(1%)	26.2(1%)
T 10	54.9	44.0(ns)	27.7(1%)	64.9(ns)
HIGH 10	61.1	50.0(ns)	28.2(1%)	35.2(ns)
TD 70	29.2	30.1(ns)	23.3(ns)	23.0(ns)

^aSignificance level for difference from >2 m, in parenthesis. Mann-Whitney U tests. ns: not significant.

TABLE 5. FLIGHT SPEED, ADVANCE SPEED, AND TORTUOSITY OF FLIGHT TRACK DURING ORIENTATION OF POTATO TUBERWORM MOTH^a

Treatment	Start to 2-m line			2-m line to lure (end)		
	Flt spd (cm/sec)	Adv spd (cm/sec)	Tort	Flt spd (cm/sec)	Adv spd (cm/sec)	Tort
FE 10	32.6ab	19.1ab	2.6a	21.0ab	7.1ab	4.0a
TD 10	35.5a	24.3a	1.9a	20.8ab	9.4ab	3.4a
T 10	40.5a	30.4a	1.4a	25.8a	14.1a	2.8a
HIGH 10	37.4a	20.7a	2.2a	21.1a	11.4ab	2.8a
TD 70	18.0b	9.4b	2.3a	11.1b	4.3b	5.7a

^aMeans followed by the same letter in the same column are not significantly different at 5% (Duncan's multiple-range test). Flt spd and Adv spd, and Tort refer flight speed, advance speed, and tortuosity, respectively.

TABLE 6. DIFFERENCES IN FLIGHT SPEED, ADVANCE SPEED, AND TORTUOSITY BETWEEN START TO 2-m AND 2-m TO LURE (END) RANGES WITH LURES SET AT 10 cm HIGH

	Start to 2 m	2 m to lure (end)	Difference ^a
Flight speed (cm/sec)	36.9	22.7	1%
Advance speed (cm/sec)	24.1	11.0	1%
Tortuosity	2.0	3.2	1%

^aMann-Whitney U tests.

2 m and 2 m to lure (end) in each treatment were not significant statistically because of large variations and small sample sizes. The data for the 10-cm-high lures were, therefore, combined before comparing the value for the two distance ranges (Table 6). All the parameters compared showed a significant difference (Mann-Whitney U test). The flight speed was reduced, and the tortuosity was increased as the males came closer to the source.

DISCUSSION

The behavior of male moths approaching a female in Lepidoptera is divided into two stages: flight from a distance and close-range behavior. The function of the distance flight is thought to be a fast approach to the vicinity of a female, and that of the close-range behavior is thought to enable the male to pinpoint the female. Continuous flight would seem to be the most effective procedure in the first stage.

In fact, many species do engage in continuous flight from a distance. This strategy may be especially adaptive in species whose habitat lies within the wide range in height of a tree. Elkinton and Cardé (1983) have shown that the catch of male *L. dispar* at the traps in the forest was evenly distributed at all heights (0–6 m). Also, height of flight in *Hyphantria cunea* is distributed over a wide range (1–6 m) (Hidaka, 1972), but it was considered questionable whether the same is true of species whose habitat is near the ground.

The approach behavior of *P. operculella*, composed of a chain of short flights as suggested by Ono (1985), differs from that of other species. This type of approach was observed with all the lures tested and even with lures set at the high position. The beeline measurement in this experiment (Figure 1) may underestimate the flight length, but flights between landings seemed to be fairly straight. The estimated length of flights shown here is, therefore, thought to be approximate, a supposition supported by the fairly high flight speed shown in

Table 5. We never saw long sustained flights during the whole series of observations.

In the present observations, the longest track observed was from the 12-m line. At least within this range, there were no changes in the flight pattern from the starting point to the 2-m line, except with the lure set at 70 cm. This suggests that the short flight type of approach observed in the range from start to about 2 m corresponds to the longer flight orientation of other moths (David et al., 1983; Kawasaki, 1981).

Ono (1985) has pointed out the adaptive significance of the close-range behavior of *P. operculella*, in which landing and walking were essential for mate finding. The approach behavior shown here is also thought to be adaptive because the habitat of *P. operculella* is on potato plants, which have dense foliage near the ground. Short flights would be more effective for finding females that are on or in this foliage than a continuous long flight, because well-defined plumes typical of unobstructed habitats may be replaced by broad active spaces enveloping the area of the plant near the female.

Males changed their behavior on arriving near a lure. The behavior in this area sometimes included jumping, walking, or walking while wing-fanning. The flight length was shortened, flight speed reduced, and the tortuosity of the track increased (Table 4 and 6). These behavioral changes represent a switch to close-range behavior. The distance at which switching occurs was similar to that observed in earlier tests (Ono, 1985).

There were clear differences among the lure treatments in the percentage of males arriving. The low percentages locating the female extracts may have been due to the low pheromone content. The pheromone content must have been very different from that of the other lures (100 female equivalents correspond to ca. 1/100 of a TD lure).

The arrival percentages at the HIGH 10 lure and lures set at 70 cm above the ground were quite low. In both cases, males would arrive near the lure but could not find the lure itself. There are a number of possible reasons for these results. With the lures set at 70 cm high, the flight length was not shortened in the 0- to 1-m range distance and the males frequently stopped further away from the source during oriented flight (Figure 5). This suggests that they missed a pheromone plume to follow, and they failed to switch to close-range behavior. With the HIGH 10 lure, there may have been an inhibitory or disrupting effect. Many males suddenly flew away on arriving within 2 m from the lure. Voerman and Rothschild (1978) have shown that there was an optimal dose of pheromone compound on a septum for trapping under field conditions. Although a decrease of trapping efficiency in their data appeared at doses 10 times higher than ours, these trends suggest that an extremely high dose of pheromone has an inhibitory or disrupting effect on male behavior.

The behavioral change occurred further away from the TD 10 lure (Table

4). This may have been due to the influence of D (diene component). The same pattern was not observed at the FE 10 lure. These two lures were not comparable, however, because of the difference in pheromone content referred to above.

Walking is an essential attribute of the final approach to the female in this species (Ono, 1985). In the present tests, males showed insufficient close-range behavior when the lure was set at 70 cm high (Table 4 and Figure 5). Males were also not caught by a trap set at that height (Table 3). Perhaps the height of 70 cm above the substrate may exceed the height limit for *P. operculella* flight in their normal life.

In these observations, behavioral responses to T (triene component) alone were similar to those with the blend lure. The role of D (diene component) is not clear from these results. Tóth et al. (1984) showed that addition of D prolonged the time spent at a lure source. Ono (1985) obtained similar results in the field, but field trapping in Nagoya failed to support these findings (Ono and Orita, 1986), suggesting that D does not have any role in flight from a distance.

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ACYCLOHEXANEDIONES FROM SETAL EXUDATE OF
HAWTHORN LACE BUG NYMPH *Corythucha cydoniae*
(HEMIPTERA: TINGIDAE)

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Abstract—The three major components of setal exudate from nymphs of the Hawthorn lace bug, *Corythucha cydoniae*, were identified as 3,6-dihydroxy-2-[1-oxo-6(*E*),8(*E*)-tetradecadienyl]cyclohex-2-en-1-one; 3,6-dihydroxy-2-[1-oxo-dodecanyl]-cyclohex-2-en-1-one; 3,6-dihydroxy-2-[1-oxo-8-dodeceny]cyclohex-2-en-1-one. An additional 10 minor components were partially characterized.

Key Words—Hawthorn lace bug, *Corythucha cydoniae*, Hemiptera, Tingidae, 3,6-dihydroxy-2-[1-oxo-6(*E*),8(*E*)-tetradecadienyl]cyclohex-2-en-1-one, 3,6-dihydroxy-2-[1-oxo-dodecanyl]-cyclohex-2-en-1-one, 3,6-dihydroxy-2-[1-oxo-8-dodeceny]cyclohex-2-en-1-one, 3,6-dihydroxy-2-acyl-cyclohex-2-en-1-ones, setal exudate.

INTRODUCTION

The nymphs of many species of lace bugs destructively feed in contiguous aggregations on the abaxial leaf surface of many trees and ornamental shrubs. In several genera of lace bugs, the nymph has numerous external secretory hairlike structures (setae) over the surface of the body and antenna that support viscous microdroplets (Livingstone, 1978). We have noted the unusually low number of reports of parasites and predators that attack lace bug nymphs, an

obvious food resource (Oliver et al., 1985), and have been examining the chemical composition of the exocrine secretions to determine their function in a suspected defense system. Species were selected from two genera with presocial behavior; one of asiatic origin and one native to North America. To date, we have reported (Oliver et al., 1985) the occurrence of 1-(2,6-dihydroxyphenyl)-1,3-diketones, along with the corresponding 2-alkyl-5-hydroxychromones and chromanones, as well as normal chain aldehydes and ketones in the setal exudate from the azalea bug (*Stephanitis pyrioides*). From the rhododendron lace bug (*S. rhododendri*) a more elaborate series of 1-(2,6-dihydroxyphenyl)-1,3-diketones and related chromones, and chromones possessing an additional oxygen, have been identified (Oliver et al., 1987). From the sycamore lace bug (*Corythucha ciliata*), the novel compound 3,6-dihydroxy-2-[1-oxo-10(*E*)-tetradecenyl]cyclohex-2-en-1-one has been isolated and identified (Lusby et al., 1987).

In this continuing examination of the major components of lace bug setal exudates, we now report the isolation and identification of three major components from the hawthorn lace bug (*C. cydoniae*) and the partial characterization of an additional 10 minor components.

METHODS AND MATERIALS

Insects were maintained in a greenhouse on either pyracantha *Pyracantha coccinea* var. *Lelandei* or Washington Hawthorn *Crataegus phaenopyrum* (1–2 m tall) grown in 35.2-liter baskets. As the insects grew to maturity, their cast molt skins, which accumulated on the leaves, were collected by aspiration into Pasteur pipets, transferred to a fritted-disk funnel, and rinsed with methanol.

Isolation of Components

By means of methanol rinses (4 × 50 ml), microdroplets of setal exudate were washed from 7.2 g of cast skins and nymphs. After removing methanol under vacuum, the residue was extracted with several portions of warm hexane (total of 35 ml), which were combined and filtered. Removal of the hexane yielded 110 mg of dark brown residue. Flash chromatography of the residue was performed on a 20% silver nitrate-loaded silica gel column (8 × 2.5 cm) using hexane saturated with formic acid (100 ml) and 10%, then 25%, ethyl acetate in hexane saturated with formic acid (250 and 400 ml, respectively); 25-ml fractions were collected. Fractions 7 and 8 provided 42 mg of a dark brown residue that contained the compounds of interest as determined by gas chromatography (GC) and gas chromatographic-mass spectrometry (GC-MS) analyses.

HPLC separation (20% silver nitrate on 20- μ m silica gel) of the above

residue yielded six fractions. Fractions 4 and 5 were combined and separated further by flash chromatography on 25 g of 20% silver nitrate on Unisil using 100, 250, and 500 ml, respectively, of 2.5%, 5.0%, and 7.5% ethyl acetate in (1:1) hexane-hexane saturated with formic acid. After collecting two initial 50-ml fractions, a series of 5.5-ml fractions was collected and their content monitored by GC. Fractions 70 and 71 contained the major component and were combined and analyzed by GC-MS, [^1H]NMR, FT-IR, and UV. An aliquot was subjected to ozonolysis. A portion of the ozonolysis products was analyzed directly by GC-MS, while the remaining portion was reacted with *O*-benzylhydroxylamine and the resultant *O*-benzyloximes analyzed by GC-MS.

Instrumentation

Mass spectra were obtained from a Finnigan model 4500 quadrupole instrument fitted with an on-column injector (J & W Scientific) and a 30-m \times 0.32-mm-ID fused silica column with a 0.25- μm DB-1 film (J & W Scientific) with the temperature held at 170°C for 2 min and then programmed to 260°C at 2°C/min. Ammonia and deuterioammonia chemical ionization (CI) spectra were obtained at an indicated source pressure of 0.6 torr and a source temperature of 80°C. A source temperature of 150°C and an ionizing voltage of 70 eV were used to produce electron ionization (EI) spectra. High-resolution mass spectral analyses were performed by the Midwest Center for Mass Spectrometry (Lincoln, Nebraska). Gas-liquid chromatographic trapping was performed on a Varian model 3700 equipped with a thermal conductivity detector and a 15-m \times 0.58-mm-ID Megabore column with a 1.5- μm film of DB-1 (J & W Scientific) and operated at 210°C. HPLC separations were achieved using a Spectra Physics model SP 8700 fitted with a 1-cm \times 22-cm 20% AgNO_3 on 20- μm silica gel column which was eluted with 95:3:2 (hexane saturated with formic acid-tetrahydrofuran-9:1 hexane-isopropanol) at 4 ml/min and observing at either 220 or 270 nm. [^1H]NMR spectra were obtained from a Nicolet NT-300 (300 MHz) Fourier transform spectrometer using either CDCl_3 and C_6D_6 as solvents and TMS as an internal standard. ^1H chemical shifts are reported in δ from TMS and coupling constants are in Hz. UV spectra, 190–400 nm, were obtained with a Perkin-Elmer model 559 using 95% EtOH as solvent. Infrared spectra were obtained from a Nicolet model 205XC GC-FTIR instrument and processed with a Nicos V3.6 operating system.

Ozonolysis and Preparation of O-Benzylloximes

Into a 1-ml conical vial was placed 10 μg (30 nmol in 10 μl CH_2Cl_2) of compound 6. After cooling to -70°C (Dry Ice- CH_2Cl_2 bath), 600 nmol O_3 (24 μl CH_2Cl_2 at -70°C saturated with O_3) was added and the vial allowed to stand at -70°C for 0.5 hr. While still at -70°C , 41 μg (600 nmol) dimethyl

sulfide (41 μl 0.1% in CH_2Cl_2) was added, and the vial allowed to come to room temperature. An aliquot of the ozonolysis reaction mixture was analyzed by EI and CI GC-MS. To one half of the ozonolysis reaction mixture (equivalent to 5 μg , 15 nmol starting material) was added 19 μg (150 nmol) *O*-benzylhydroxylamine (25 μl aq. solution at pH 4.5). After standing, with occasional shaking, at room temperature for 1 hr, 10 μl 2 M HCl was added and the vial shaken. The CH_2Cl_2 layer was analyzed by EI and CI GC-MS. Compound 1 was examined in a similar manner.

Hydrogenation

An aliquot of a fraction from flash chromatography was dissolved in 0.2 ml *N,N*-dimethylformamide (DMF) saturated with $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$. While stirring vigorously at ambient temperature, 0.05 ml of 0.5 M NaBH_4 in DMF was added and approximately 10 sec later a black precipitate formed. A second 0.05-ml portion of the NaBH_4 was added, and after 1 min the reaction was quenched with aqueous NH_4Cl , and filtered through a pad of Celite. The filtrate was passed through a C_{18} extraction cartridge that was subsequently rinsed with water. The products were eluted from the cartridge with tetrahydrofuran, concentrated, and analyzed by GC-MS.

Physical Data

Compound 1 (3,6-Dihydroxy-2-[1-oxo-8-dodeceny]cyclohex-2-en-1-one). EI-MS *m/z*: 308(100) M^+ , 290(2)[$\text{M}-\text{H}_2\text{O}$] $^+$, 264(7)[$\text{M}-\text{C}_2\text{H}_4\text{O}$] $^+$, 219(13), 208(33), 183(86), 170(40), 168(52), 155(30), 142(24), 137(30), 126(44), 69(35), 55(72); CI(NH_3)-MS *m/z*: 326(100)[$\text{M} + \text{NH}_4$] $^+$, 309(19)[$\text{M} + \text{H}$] $^+$; CI(ND_3)-MS *m/z*: 332(100)[$\text{M} + \text{ND}_4$] $^+$, 312(21)[$\text{M} + \text{D}$] $^+$.

Ozonolysis Product from Compound 1 [3,6-Dihydroxy-2-(1,8-dioxooctyl)-2-cyclohexen-1-one]. EI-MS *m/z*: 268(3) M^+ , 240(63), 196(32), 183(100), 170(23), 153(54), 139(59), 95(68), 69(72), 55(83); CI(NH_3)-MS *m/z*: 303(4)[$\text{M} + (\text{NH}_3)_2\text{H}$] $^+$, 286(100)[$\text{M} + \text{NH}_4$] $^+$, 269(3)[$\text{M} + \text{H}$] $^+$.

O-Benzylloximes from Ozonolysis of Compound 1: O-Benzyloxime 1 (*n*-Butyraldehyde-*O*-Benzyloxime). EI-MS *m/z*: 177(2) M^+ , 160(5), 147(9), 105(21), 91(100), 77(10), 65(10); CI(NH_3)-MS: 212(79)[$\text{M} + (\text{NH}_3)_2\text{H}$] $^+$, 195(100)[$\text{M} + \text{NH}_4$] $^+$, 178(12)[$\text{M} + \text{H}$] $^+$.

O-Benzyloxime 2 [3,6-dihydroxy-2-(1,8-dioxooctyl)-2-cyclohexene-1-one-*O*-benzyloxime]. EI-MS *m/z*: 225(3), 183(4), 108(10), 91(100), 79(12), 77(11), 55(10); CI(NH_3)-MS: 391(100)[$\text{M} + \text{NH}_4$] $^+$, 374(9)[$\text{M} + \text{H}$] $^+$.

Compound 2 (3,6-Dihydroxy-2-[1-oxo-dodecanyl]cyclohex-2-en-1-one). EI-MS *m/z*: 310(42) M^+ , 292(3)[$\text{M} - \text{H}_2\text{O}$] $^+$, 266(22)[$\text{M} - \text{C}_2\text{H}_4\text{O}$] $^+$, 183(100), 170(27), 168(20), 153(21), 140(28), 126(16), 55(23); CI(NH_3)-MS:

328(100)[M + NH₄]⁺, 311(52)[M + H]⁺; CI(ND₃)-MS: 334(100)[M + ND₄]⁺, 314(54)[M + D]⁺.

Compound 6 (3,6-Dihydroxy-2-[1-oxo-6(E),8(E)-tetradecadienyl]-cyclohex-2-en-1-one). UV (nm, 95% EtOH); λ_{max} 229(ε ca. 45,000), 270(ε ca. 15,000). IR (cm⁻¹, gas phase); 3017(w), 2968(m), 2934(s), 2885(m), 1679(s), 1562(s), 1481(s), 1421(m), 1392(m), 1919(m), 984(m); EI-MS *m/z*: 334(5)M⁺, 316(100)[M - H₂O]⁺, 290(31)[M - C₂H₄O]⁺, 245(26), 219(29), 205(98), 183(34), 170(69), 155(36), 142(29), 137(32), 126(55), 79(67), 67(75), 55(53); HRMS *m/z*: 334.2144, M⁺ (C₂₀H₃₀O₄, Calc. 334.2144); 316.2035, [M - H₂O]⁺ (C₂₀H₂₈O₃, Calc. 316.2038); 290.1885, [M - C₂H₄O]⁺ (C₁₈H₂₆O₃, Calc. 290.1882); CI(NH₃)-MS *m/z*: 352(100)[M + NH₄]⁺, 335(32)[M + H]⁺; CI(ND₃)-MS *m/z*: 358(100)[M + ND₄]⁺, 338(28)[M + D]⁺; [¹H]NMR(CDCl₃); 0.84 (3H, t, *J* = 7, CH₃), 1.26–1.32 (6H, m, alkane CH₂), 1.44 (2H, m, CH₂), 1.56–1.69 (2H, m), 1.70–1.85 (H, m), 2.02 (4H, m), 2.33 (H, m), 2.33 (OH), 2.78 (2H, m), 3.01 (2H, m), 4.08 (H, dd, *J* = 13 and 4Hz), 5.57 (2H, m), 5.98 (2H, m), 18.20 (OH).

Ozonolysis Product from Compound 6 [3,6-Dihydroxy-2-(1,6-dioxohexyl)-2-cyclohexen-1-one]. EI-MS *m/z*: 212(39), 194(31), 183(62), 168(57), 153(55), 140(56), 126(28), 85(55), 67(100), 55(95); CI(NH₃)-MS *m/z*: 258(100)[M + NH₄]⁺, 241(4)[M + H]⁺.

O-Benzylloximes from Ozonolysis of Compound 6: O-Benzylloxime 3 (Hexanal-O-benzylloxime). EI-MS *m/z*: 205(1.2)M⁺, 188(0.7), 175(0.8), 149(8), 91(100), 77(5), 65(5); CI(NH₃)-MS: 240(54)[M + (NH₃)₂H]⁺, 223(100)[M + NH₄]⁺, 206(19)[M + H]⁺.

O-Benzylloxime 4 (Glyoxal-O-benzylloxime). EI-MS *m/z* 268(3)M⁺, 181(4), 161(2), 145(1), 105(2), 91(100), 77(8), 65(7); CI(NH₃)-MS: 303(2)[M + (NH₃)₂H]⁺, 286(100)[M + NH₄]⁺.

O-Benzylloxime 5 [3,6-dihydroxy-2-(1,6-dioxohexyl)-2-cyclohexen-2-one-O-benzylloxime]. EI-MS *m/z*: 345(0.5)M⁺, 221(1), 193(1), 183(2), 137(2), 108(6), 91(100), 77(8), 69(7), 55(7); CI(NH₃)-MS: 363(100)[M + NH₄]⁺, 346(3)[M + H]⁺.

RESULTS AND DISCUSSION

Initially, samples of setal exudate material were obtained by wicking the microdroplets onto small triangular pieces of absorbent filter paper. Samples obtained in this manner will be referred to as “wicked” samples. Examination by GC-MS of a dichloromethane extract of the filter paper revealed a remarkably clean sample consisting of a band of peaks (Figure 1) containing 13 components of retention times from 14 to 33 min and ranging in molecular weight

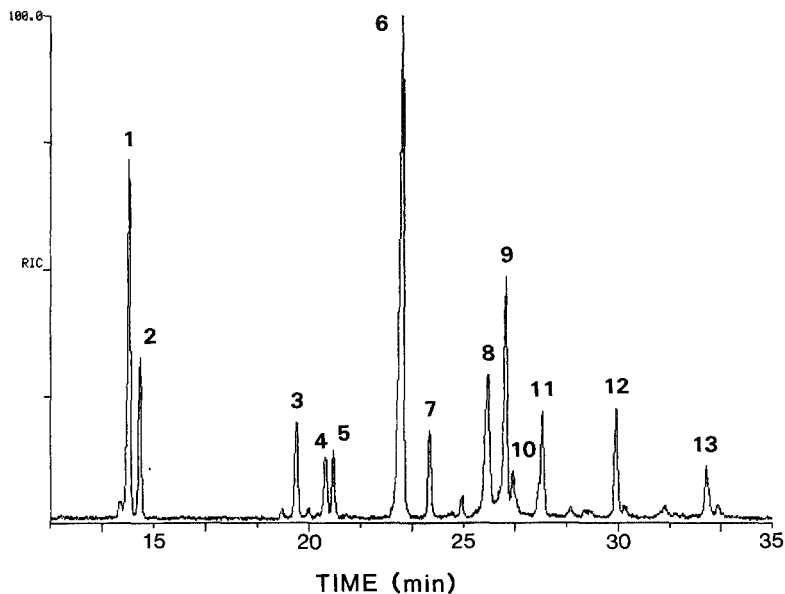


FIG. 1. Reconstructed total ion chromatogram of setal exudate compounds from *C. cydoniae*.

from 308 to 378 (Table 1). By comparing the value for the ammonium adduct ion using first ammonia then deuterioammonia CI-MS, it was determined that all of the components possessed two exchangeable hydrogens except compound 7, which had one. Electron ionization mass spectra, while being distinct from one another, were sufficiently similar to suggest the components of the mixture were structurally related.

Examination of cast skins revealed retention of setal microdroplets; therefore, in order to secure sufficient material for additional spectroscopic characterization, lace bugs were mass reared and cast skins collected. Extracts of cast skins yielded material that contained fewer spurious compounds than material obtained from extraction of intact nymphs, but the cast skin extract was not as clean as wicked samples. Gas chromatographic and mass spectral analyses of exudate wicked from insects reared on either hawthorn or pyracantha were indistinguishable, and therefore cast skins from both sources were pooled.

The isolation procedure yielded a sufficient amount of compound 6 to permit both [^1H]NMR analysis and ozonolysis followed by preparation of *O*-benzylloxime derivatives. However, only enough compound 1 was obtained to permit ozonolysis and subsequent formation of *O*-benzylloximes. No other components were individually isolated.

TABLE 1. CHARACTERISTICS OF SETAL EXUDATE COMPONENTS OF *C. cydoniae* NYMPHS

Compound number	Percent ^a	Ret time ^b	Molecular weight	H _{ex} ^c	R ^d	Ring moiety ^e
1	14.4	14.2	308	2	12/1	I
2	6.0	14.6	310	2	12/0	I
3	4.6	19.6	334	2	14/2	I
4	3.0	20.5	336	2	14/1	I
5	3.2	20.8	336	2	14/1	I
6	28.1	23.7	334	2	14/2	I
7	3.6	23.9	346	1	16/2	II
8	9.8	25.8	350	2		
9	11.2	26.4	362	2	16/2	I
10	2.3	26.6	362	2	16/2	I
11	5.1	27.6	364	2	16/1	I
12	5.2	29.9	362	2	16/2	I
13	3.5	32.9	378	2		

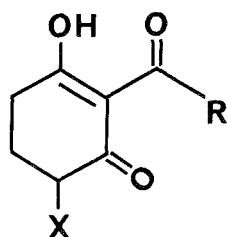
^aPercentage composition based on the 13 most abundant components as measured by reconstructed ion chromatogram of electron ionization mass spectral analysis.

^b30-m DB-1 fused silica column (see Methods and Materials for details).

^cNumber of exchangeable hydrogens.

^dLength of side chain/number of double bonds in side chain.

^eSee Figure 2 for structure of ring moiety.



TYPE	X
I	OH
II	H

FIG. 2. Structures of setal exudate compounds from *C. cydoniae* (R defined in Table 1).

Compound 6

Examination of compound 6, the most abundant component, by [¹H]NMR yielded a spectrum nearly identical to that reported for 3,6-dihydroxy-2-[1-oxo-10(*E*)-tetradecenyl]-cyclohex-2-en-1-one that had been isolated from *Corythuca cilata* (Lusby et al., 1987). The near identity of the [¹H]NMR spectra together with both HRMS and CI-MS established that compound 6 contains the ring moiety shown in Figure 1 and possessed a side chain of 14 carbons containing two olefinic bonds. In order to locate the position of the olefinic bonds within the side chain, a portion of compound 6 was subjected to ozonolysis.

Ozonolysis of Compound 6. Mass spectral analyses of the ozonolysis products from component 6 indicated the formation of 3,6-dihydroxy-2-(1,6-dioxohexyl)-2-cyclohexen-1-one, thereby locating one of the olefinic bonds of 6 between carbons 6' and 7' of the side chain. Further examination of the mass spectral data of the ozonolysis reaction did not provide the identity of other products, which would locate the position of the second olefinic bond.

Reaction of the ozonolysis products with *O*-benzylhydroxylamine yielded three oximes, one of which corresponded to the above-identified aldehyde. A second oxime compound was derived from hexanal, indicating cleavage of a six-carbon fragment from the end of the side chain. The third oxime, a dioxime, yielded a multiplicity of gas chromatographic peaks due to combination of *syn* and *anti* configurations. Their mass spectra and a gas chromatographic comparison to an authentic sample established that they were derived from glyoxal. These data indicate the presence of a conjugated diene system with olefinic bonds located at carbons 6' and 8' of the side chain. Among the [¹H]NMR signals were two in the olefinic region: a multiplet at 5.98 δ and a multiplet at 5.57 δ. Irradiation at 5.57 δ collapsed the signal at 5.98 to a singlet, and irradiation at 5.98 δ yielded a triplet at 5.57 δ. These data confirm the presence of a conjugated diene system.

In order to establish isomeric configuration of the side-chain olefinic bonds, GC-FTIR analyses were performed on compound 6 and, as models, the four isomers of hexadeca-5,7-dienyl acetate. A band at 984 cm⁻¹, corresponding to C—H out-of-plane stretching, was present in both compound 6 and the *E,E* isomer of hexadeca-5,7-dienyl acetate but was absent in the remaining three isomers. A like band has been previously reported in the spectrum of the *E,E* isomer during the comparison of the four isomers of hexadeca-10,12-dien-1-ol (Butenandt et al., 1962).

The UV spectrum exhibited maxima at 229 nm (ε ca. 45,000) and 270 nm (ε ca. 15,000), with slight inflections at 216 and 235 nm (determined by inspection of the first derivative), and a shoulder at 226 nm. By comparison to a similar compound (Lusby et al., 1987) possessing a single olefinic bond in the side chain, it is seen that the conjugated diolefinic system in compound 6 produces both a greater extinction coefficient at the 229 nm maximum and a shift

of that maximum to slightly shorter wavelength. The above data establish compound 6 as 3,6-dihydroxy-2-[1-oxo-6(*E*),8(*E*)-tetradecadienyl]cyclohex-2-en-1-one. The configuration of the chiral center at C-6 was not determined.

Compound 2

Compound 2, which has a molecular weight of 310 and two exchangeable hydrogens, was identical in both GC retention time and mass spectrum to 3,6-dihydroxy-2-(1-oxododecanyl)-cyclohex-2-en-1-one, whose structure was recently confirmed by synthesis (Oliver and Lusby, 1988).

Compound 1

Compound 1 possesses a molecular weight of 308, thereby indicating a side-chain length of 12 carbons with a single olefinic bond. Hydrogenation of compound 1 yielded compound 2, thereby establishing the structure of the ring moiety. In order to determine the location of the olefinic bond in the side chain, compound 1 was subjected to ozonolysis and, under the gas chromatographic conditions used, a single reaction product was observed. Ammonia CI mass spectral analysis yielded a molecular weight of 238. EI analysis indicated the product to be 3,6-dihydroxy-2-(1,8-dioxooctyl)-2-cyclohexen-1-one. This indicated the presence of an olefinic bond between carbons 8' and 9' of compound 1. The ozonolysis products were converted to *O*-benzylloximes by reaction with *O*-benzylhydroxylamine. CI and EI mass spectral analysis of the *O*-benzylloxime reaction mixture revealed the presence of the *O*-benzylloxime of *n*-butyraldehyde, thus demonstrating the presence of *n*-butyraldehyde in the original ozonolysis product, and thereby confirmed the location of the olefinic bond. Insufficient material was available to determine the geometry of the double bond.

Compounds 3-5, 7-12

A fraction containing all components of the wicked sample except compounds 8 and 13 was hydrogenated using NaBH₄/NiCl₂/DMF. Catalytic hydrogenation with Pd or Pt catalysts provided results that were difficult to reproduce as well as to interpret. In contrast, the presumed *in situ* generation of both hydrogen and catalyst achieved by adding NaBH₄ to DMF containing NiCl₂ afforded clean reduction of olefinic side chains without affecting the enolic C=C or other functional groups. Interestingly, this same medium had been used recently to selectively reduce an isoxazole N—O bond in the presence of a side chain C=C (Oliver and Lusby, 1988). Slight modification of the reaction conditions (Methods and Materials) allowed, in this case, clean reduction of the side-chain double bonds while preserving the ring functionalities. Compounds 3, 4, 5, and 6, with molecular weights of 334, 336, 336, and 334 respectively, were hydrogenated to a single product with a molecular weight of 338 and

having a mass spectrum virtually identical to that of compound 2 except for the molecular ion and ions derived by simple losses from the molecular ion. In a like manner, compounds 9, 10, 11, and 12 yielded a single product with a molecular weight of 366 and a saturated side chain. These data indicate that compounds 1–6 and 9–12 all possess the same ring moiety but differ by length of the side chain (12, 14, or 16 carbons) and/or degree and position of unsaturation in the side chain.

These results demonstrate the presence of exudate compounds in *C. cydoniae* that are very similar to those isolated from *C. ciliata* (Lusby et al., 1987) and quite distinct from those isolated from *S. pyrioides* (Oliver et al., 1985) and *S. rhododendri* (Oliver et al., 1987).

Compounds from the two genera are distinct in that those from *Corythucha* are nonaromatic vs. the aromatics from *Stephanitis*; however, the gross structures and oxygenation patterns suggest related biosynthetic pathways.

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ACIDIC FOG-INDUCED CHANGES IN HOST-PLANT SUITABILITY

Interactions of *Trichoplusia ni* and *Phaseolus lunatus*

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Abstract—*Phaseolus lunatus* L. (Henderson Bush lima beans) were exposed to 2 hr acidic fogs with 2.5:1.0 (v/v) nitrogen-sulfur ratio typical of the west coast of the United States. Fogs with pH values of 2.0 ($P < 0.01$, t tests), 2.5 ($P < 0.05$), or 3.0 ($P < 0.01$) increased percent total nitrogen (dry weight) of foliage as compared to plants subjected to control fogs with a pH of 6.3–6.5. Fresh weight concentrations of soluble protein and certain free amino acid concentrations were increased by plant exposure to acidic fogs with a pH of 2.5 (t tests, $P < 0.05$). Concentrations of free amino acids considered essential for insect growth, as well as nonessential and total free amino acids were not significantly affected by any treatment ($P > 0.05$, t test). Water content (%) of foliage was not changed significantly ($P > 0.05$, t test) by exposure to any of the fogs. *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) larvae ate significantly more foliage and gained significantly more weight on plants treated with 3.0 pH fogs ($P < 0.01$, t test). Several potential explanations are offered for the lack of significant weight gain by larvae on plants in which soluble protein levels, free amino acid concentrations, or percent total nitrogen contents were enhanced by acidic fogs with a pH of 2.5 and 2.0. No larval feeding preference was detected for foliage exposed to acidic versus control fogs, and no significant differences were detected in percent survival of *T. ni* eggs exposed to acidic or control fogs. Some implications of acidic fogs for population dynamics of *T. ni* are discussed.

Key Words—Acidic fog, *Phaseolus lunatus*, *Trichoplusia ni*, cabbage looper, Lepidoptera, Noctuidae, nitrogen, free amino acid, soluble protein, plant-insect interactions, air pollution.

INTRODUCTION

Like the gaseous photochemical oxidants, acidic fogs can have beneficial as well as detrimental consequences for plants. Wet deposition of pollutants (rain, fog, dew) on foliar surfaces either can act as fertilizers or cause a sequence of cellular collapse resulting in necrotic lesions (Shriner, 1986). Previous reports noted deleterious effects such as lesion development, weathering of cuticular wax, foliar leaching, and modification of physiological responses (Cowling, 1982; Lintherst et al., 1982). These effects have been duplicated with short-term exposure to acidic fogs of pH 2.3 or lower under laboratory conditions (Granett and Taylor, 1981; Granett and Musselman, 1984). Because ambient fogs in the Los Angeles Basin exhibit an acidity of pH 1.69–3.0, significant foliar necrosis leading to yield losses in crop plants is not unusual (Hoffman, 1984).

Physiological changes in plants affected by air pollutants significantly alter the nutritional suitability of such plants for insect herbivores (Jones and Coleman, 1988). Substantial modifications in the form and content of nitrogen (e.g., both increases and decreases) have been reported following plant exposure to ozone and other pollutants (Chang, 1971; Mudd and Freeman, 1977). In tomatoes, ozone effects include an increase in free amino acids and soluble proteins (Trumble et al., 1987), which may be more readily assimilated by insects than the nitrogen bound in the structural components of cells. One objective of the study reported here was to determine if plants subjected to acidic deposition and damaged by acidic fogs develop different total nitrogen, soluble protein, and free amino acid profiles than plants exposed to nonacidic fogs.

The implications of increased concentrations of assimilable forms of nitrogen for insect herbivore development are considerable (Strong et al., 1984; White, 1984). Nitrogen availability dramatically affects key life processes such as growth rates, survival, and reproductive capacity (Onuf, 1978; Prestidge, 1982; Prestidge and McNeill, 1983). Because air pollutants in general, and acidic fogs in particular, often occur over broad geographic areas (Lee, 1982; Hoffman et al., 1985), the cumulative effects on herbivores at the population level may be of substantial importance (White, 1984). Thus, potential changes in plant physiology due to stress or direct injury from air pollutants may well have more serious consequences than are indicated when the plant system is examined in the absence of herbivores.

At present, little research has focused on the effects of acidic fogs on the nutritional suitability of plants for insect herbivores. Although several studies have, in some cases, documented an increase in food consumption or preference for foliage exposed to ozone or SO₂ (Hughes et al., 1981; Jeffords and Endress, 1984; Endress and Post, 1985; Trumble et al., 1987; Coleman and Jones, 1988; Jones and Coleman, 1988), these are by no means the only responses noted

(Jones and Coleman 1988). The impact on herbivores of foliar deposition of nitrates associated with high-nitrogen acidic fogs and potential damage or stresses associated with acidic fogs have not been reported. Therefore, a second objective of our research was to document the potential influence of acidic fogs on some key factors influencing herbivore population dynamics.

METHODS AND MATERIALS

The Plant-Insect System. Henderson bush lima beans, *Phaseolus lunatus* L., used in the experiments were germinated in the greenhouse in UC Soil Mixture (Matkin and Chandler, 1957) and fertilized twice weekly with one-half strength Hoagland's nutrient solution (Downs and Hellmers, 1975). Thus, all plants were provided with adequate nitrogen, similar to what would occur in an agricultural setting. Although populations of nitrogen-fixing symbiotes were not surveyed, the provision of adequate nitrogen and the use of washed seed and sterilized soil probably minimized the potential for differential impact due to symbionts between treatments. All plants within a test were from the same seedlot, the same age (4-5 weeks old), and had the primary and first trifoliolate leaves fully expanded.

For the nitrogen studies, 10 pairs of plants within each pH level of fog were further standardized by CO₂ assimilation rates using a Li-Cor 6000 photosynthesis measurement system (Li-Cor Inc., Lincoln, Nebraska). Nondestructive measurements were recorded 20 hr pretreatment in the laboratory under a metal halide lamp providing a minimum of 1000 $\mu\text{E}/\text{sec}\cdot\text{m}^2$. After sampling the primary leaves of ca. 40 plants, 10 pairs of plants were identified that differed in CO₂ assimilation by less than 0.003 mg CO₂/sec $\cdot\text{m}^{-2}$. One of each pair of plants with comparable CO₂ assimilation rates was exposed to a control fog, and the other to an acidic fog. Standardizing test plants by CO₂ assimilation rates is important, because even plants from the same seedlot grown concurrently can differ significantly in photosynthetic and physiological activity (Trumble et al., 1985), and CO₂ assimilation rates are proportionally related to percent nitrogen and soluble protein levels in plants (Hesketh et al., 1983; and references therein).

Cabbage looper, *Trichoplusia ni* (Hübner), eggs and larvae were obtained from a laboratory colony reared on artificial diet (Shorey and Hale, 1965). The colony was maintained at a photoperiod of 12:12 hr light-dark and a temperature of $21 \pm 1^\circ\text{C}$. Although larvae fed on an artificial diet may not respond to stimuli exactly the same in all situations as foliage-fed larvae, this approach did provide standardized experimental organisms with regard to age, stress, and nutritional backgrounds.

Simulated Acidic Fogs. Simulated acidic fogs were prepared by adjusting

distilled water to pH 2.0, 2.5, and 3.0 with reagent-grade nitric and sulfuric acid mixed at a 2.5:1 (v/v) ratio. This mixture is typical of the high-nitrate, low-sulfate fogs in California, but lacks other ionic components of ambient moisture (Waldman et al., 1982). Control fogs consisted of distilled water with a pH of 6.3–6.5. The simulated fogs were created within a 1-m² chamber using a fogging apparatus designed by Musselman et al. (1985), which produces a droplet size averaging 20 μm in diameter. Test plants were fogged for 2 hr and then placed outdoors to dry for at least 1–2 hr.

Total Nitrogen, Soluble Protein, and Free Amino Acid Analyses. Ten pairs of plants were analyzed for each pH level of the acidic fog. After air drying, the primary leaves and an upper trifoliate leaf were excised, combined, and then divided into aliquots for analyses of total nitrogen, soluble proteins, and free amino acids. Samples were weighed immediately after collection, frozen in liquid nitrogen, and stored in an ultracold freezer (-65°C). Enough leaf material was available on each plant to allow two samples of each of the variables to be analyzed. Potential long-term changes in plant chemistry were not addressed by this study.

Total nitrogen was analyzed using the micro-Kjeldahl technique (McKenzie and Wallace, 1954). The technique was modified by replacing the mercuric oxide catalyst with copper sulfate, and by utilizing bromocresol green in place of methylene blue as an indicator. Percent water content of the foliage was determined by subtracting the dry weight (oven-dried at 70°C for 72 hr) from the fresh weight, dividing by the fresh weight, and multiplying by 100.

The technique of Jones et al. (1988) was used to quantify soluble protein. Frozen samples (0.4–0.6 g fresh weight) were ground and extracted with 10 ml 0.1 M NaOH for 30 min at room temperature. Leaf tissue was removed by centrifugation (11,500g for 10 min) and the decanted supernatant was brought to 10 ml with 0.1 M NaOH. Protein concentration in the NaOH solution was measured with the Bradford (1976) reagent using ribulose 1,5-biphosphate carboxylase (RuBPase) (Sigma Chemical Co., St. Louis, Missouri) as the standard. Samples were diluted when necessary to avoid deviations from linearity due to NaOH at high protein concentrations. Duplicate readings were made on each extraction. Values are reported as milligrams RuBPase equivalent protein per gram fresh weight of foliage.

Free amino acids were extracted using procedures modified from Hare (1983). Twenty amino acids, including a nonprotein amino acid (gamma aminobutyric acid) and two secondary amino acids (hydroxyproline and proline) were quantified by reverse-phase HPLC on a Beckman model 332 liquid chromatograph with a fluorescence detector after precolumn *o*-phthalaldehyde (OPA) derivatization using methods described by Cooper et al. (1984). A Beckman 3- μm Ultrasphere-XL ODS column 4.6 mm ID \times 70 mm in length was used to improve resolution. Prior to injection, 200 μl of 1.3 M sodium phosphate (pH

3.5) was added to reduce the pH of the sample in order to improve column life (Sista, 1986). Quantities of each amino acid were calculated from their peak areas in relation to the peak area and known quantity of the appropriate internal standard (Cooper et al., 1984).

Data on nitrogen and water contents of treated and control plants were analyzed with paired and unpaired *t* tests. Paired *t* tests were included because CO₂ assimilation rates are proportionally related to percent nitrogen and soluble protein levels in plants (Hesketh et al., 1983). Unpaired *t* tests were presented for comparative purposes because no clear relationships between either free amino acid or water content and CO₂ assimilation rates have been established. Free amino acid analyses were performed on each amino acid individually, all combined, and grouped by the "essential" vs. "nonessential" criterion of Taylor and Medici (1966).

Influence of Acidic Fogs on Insect Developmental Parameters. In order to document the direct effects of acidic fogs on survival of eggs, caged moths were allowed to oviposit for 8 hr on 4- to 5-week-old *P. lunatus*. Plants with 20 eggs developing to the point of head capsule visibility were assigned randomly to control fogs or acidic fogs with a pH of 2.0 or 3.0. A control accompanied each pH level of fog because these tests were not concurrent. Six replicates of 20 eggs were tested at each pH level. Plants with eggs were exposed to control and acidic fogs for 2 hr and then held at $26.7 \pm 1^\circ \text{C}$, $65 \pm 5\%$ relative humidity and a photoperiod of 16:8 hr light-dark until eclosion. Eggs were monitored at 24-hr intervals for at least five days. Comparisons between treatment and control groups were made with unpaired *t* tests.

Food consumption by *T. ni* larvae on plants exposed to fogs with pH values of 2.0, 2.5, and 3.0 was quantified by individually caging a neonate larva on foliage of 30 treated and 30 control plants (one larva per plant) ca. 2 hr after fogging. All larvae were treated concurrently on plant material prior to transfer to cages on the test plants. Plants and larvae were randomly selected for each treatment. A control fog was conducted simultaneously for fogs with pH values of 2.0 and 2.5 + 3.0 because logistics precluded running all acidic fogs concurrently. Plants and larvae were maintained at room temperature, which ranged from 20 to 26°C. Larvae were held until any leaf in the test began to senesce (12–13 days, at which time larvae were in the third instar). All larvae then were removed and the test was concluded. The test was terminated at this time because attempts to relocate the larvae to undamaged leaves were unsuccessful, and substantial mortality resulted. Immediately after larvae were removed, test and control leaves were photocopied and the leaf area with and without excision of feeding damage was measured on a Li-Cor 3000 leaf area meter. Differences between the measurements provided the leaf area consumed per larva. To account for potential differences in specific leaf weight at 7 and 14 days post-treatment, 20 plants were treated with 2.0 pH acidic fog as described earlier

and compared for specific leaf weight (fresh weight) with 20 control plants treated with a pH 6.3–6.5 fog (10 per sample date). Six to eight 22-mm-diameter cores (No. 14 cork borer) were taken from each expanded primary leaf (at least 70% of the total leaf available) and the fresh weight measured. This information was necessary to calculate differences in consumption rate based on leaf area if specific leaf weight varied between treatments.

Larval weight gain on foliage treated with acidic (pH 2.0 or 3.0) or control fogs was measured by individually caging 15 neonate larvae per treatment on plants in the same fashion as the food consumption test. Plants and larvae were randomly selected for each treatment. When any leaf was ca. 90% eaten or began to senesce (as evidenced by onset of yellowing), the surviving larvae (third instars) were removed and weighed. Data were analyzed by unpaired *t* test.

Preference of *T. ni* larvae for foliage exposed to acidic fogs or control fogs was evaluated using a leaf disk bioassay technique. After random selection for treatment, plants were exposed to acidic fogs with a pH of 2.0 or 3.0 (nonconcurrently), or control fogs, and allowed to dry for 3 hr before 22-mm-diameter leaf disks (No. 14 cork borer) were cut from expanded primary leaves. Two control and two test disks from individual plants (total 20 plants per treatment) were placed on moistened filter paper at alternating cardinal points in 8 × 2-cm Petri dishes. Two larvae reared to third instar on artificial diet then were placed centrally in the Petri dish and allowed to feed for 23–24 hr. This test was replicated 20 times for each acidic fog and control fog tested. The remaining leaf area of the disks was measured with a Li-Cor 3000 leaf area meter. All Petri dish experimental units contained feeding damage. Differences in leaf area consumed were analyzed with a paired *t* test (treated versus control disks within a Petri dish) to remove variability in feeding rates between larvae.

RESULTS AND DISCUSSION

Total Nitrogen, Soluble Protein, and Free Amino Acid Analyses. The total nitrogen content of *P. lunatus* exposed to acidic fogs was substantially altered as compared to control plants (Table 1). Plants exposed to fogs with increasing acidity had significantly higher nitrogen content as compared to their respective controls ($P \leq 0.1, 0.05, 0.01$, for fogs of pH 3.0, 2.5, 2.0, respectively). This increase was not linear, suggesting a nonuniform uptake. Presumably at least some of the additional nitrogen was deposited by the acidic fog in the form of nitrate and was not converted on the leaf surface to a form usable by the plant. However, considerable evidence is available, which suggests that growth of plants exposed to acidic fogs may be enhanced by the increased availability of nitrogen and sulfur (Ferenbaugh, 1976; Irving, 1979; Shriner, 1986). The sig-

TABLE 1. TOTAL NITROGEN, SOLUBLE PROTEIN AND WATER CONTENT IN *Phaseolus lunatus* EXPOSED TO FOGS OF VARYING ACIDITY^a

Source ^b	pH of test fog					
	2.0		2.5		3.0	
Percent total nitrogen (dry wt)	<i>df, t value</i>			<i>df, t value</i>		
Control plants	2.01	18, 4.257**	2.39	18, 2.488*	3.89	18, 1.904 ^c
Test plants	2.68		2.63		4.64	
mg/g fresh wt of RuBPase equivalent protein	<i>df, t value</i>			<i>df, t value</i>		
Control plants	10.64	16, -1.046	8.57	18, 3.522**	9.63	16, 0.248
Test plants	9.41		12.75		9.89	
Percent water content	<i>df, t value</i>			<i>df, t value</i>		
Control plants	86.8	18, 1.766	84.0	18, -0.525	87.4	18, 1.000
Test plants	85.8		84.3		87.9	

^a Analysis by unpaired and paired *t* tests, results were similar with both tests, so only the unpaired values were reported. Values presented in percent were subjected to arcsine square root transformation prior to analysis. Samples from paired comparisons based on 9-10 pairs of plants exhibiting similar photosynthetic rates (± 0.003 mg CO₂/sec/m²). Plant material was collected approximately 4 hr after fogging. **significance at $P < 0.01$ level, * $P < 0.05$.

^b Control plants fogged with distilled water with a pH of 6.3-6.5.

^c Significant at $P < 0.073$ level.

nificant ($P < 0.01$) increase in soluble protein concentrations for plants exposed to fogs with a pH of 2.5 suggests that some of the nitrate nitrogen responsible for the increase in total nitrogen was assimilated even within the 2-3 hr following fogging. If the increase in soluble protein was simply a response to stress, as has been demonstrated for other environmental factors (Beckerson and Hofstra, 1979; White, 1984), then foliage exposed to the 2.0 fogs also should have developed elevated concentrations. Fogs with pH values of 3.0 or below (i.e., more acidic) are well within the range shown to cause both visible and physiological damage to legumes (Shriner, 1986).

Although significant differences in concentrations of specific amino acids were observed in *P. lunatus* subjected to acidic fogs with a pH of 2.5 as compared to plant material from control fogs, no significant differences in total free amino acids, insect-essential or nonessential amino acid concentrations were evident (Table 2). Interestingly, all of the specific amino acids that showed significant changes in concentration actually increased in concentration. This is in contrast to the impact of another air pollutant, ozone, on the tomato system, where concentrations of certain amino acids were reduced (Mudd and Freeman, 1977; Trumble et al., 1987). No significant differences were observed for specific or total amino acid concentrations between plants exposed to control fogs

TABLE 2. INFLUENCE OF ACIDIC FOG OF pH 2.5 ON FREE AMINO ACID CONCENTRATIONS IN *Phaseolus lunatus*

Amino acid	Fresh weight ($\mu\text{g/g}$)		Paired <i>t</i> test ^a		Unpaired <i>t</i> test ^b	
	Control	Acidic fog	<i>P</i>	<i>t</i>	<i>P</i>	<i>t</i>
Aspartic acid	210.50	299.55	0.01	3.07	0.03	2.75
Glutamic Acid	397.26	473.14	0.03	2.80	0.10	1.75
Glutamine	19.50	43.39	0.03	2.80	0.03	2.46
Threonine	38.35	47.83	ns ^c	1.82	0.05	2.12
Valine	21.91	32.90	0.01	3.80	0.01	3.91
Essential AA	910.48	742.66	ns	-1.44	ns	0.84
Nonessential AA	2487.06	2715.68	ns	0.96	ns	0.72
Total AA ^d	3525.50	3575.40	ns	0.17	ns	0.12

^aSamples for paired comparisons from 10 pairs of plants exhibiting initially similar photosynthetic rates ($\pm 0.003 \text{ mg CO}_2/\text{sec}/\text{m}^{-2}$). Photosynthesis data collected 24 hr pretreatment under metal halide light providing a minimum of $1,000 \mu\text{E}/\text{sec}/\text{m}^{-2}$. Control plants fogged with distilled water with a pH of 6.3-6.5.

^bUnpaired *t* test, *df* = 18.

^cns = not significant at the *P* < 0.10 level.

^dIncludes the nonprotein amino acid, gamma-amino butyric acid.

or acidic fogs with a pH of 2.0 or 3.0 and are not reported. Thus, the extensive quantitative changes in amino acid levels associated with physiological effects of other air pollutants (e.g., ozone) (Menzel, 1971; Craker and Starbuck, 1972) did not occur consistently (i.e., at all pH levels) in our study.

Influence of Acidic Fogs on Insect Developmental Parameters. There were no significant differences in specific leaf weight between treatments at either 7 (means \pm SD = $6.7 \pm 0.5 \text{ mg}/\text{cm}^2$ for controls and $7.0 \pm 0.6 \text{ mg}/\text{cm}^2$ for pH 2.0; *P* > 0.05, *t* test) or 14 days posttreatment (means \pm SD = $7.2 \pm 0.5 \text{ mg}/\text{cm}^2$ for controls and $7.1 \pm 0.5 \text{ mg}/\text{cm}^2$ for pH 2.0; *P* > 0.05, *t* test), so consumption has been reported as leaf area consumed.

Exposure of host foliage to the low and moderately acidic fogs chosen (pH 3.0 and 2.5) had a significant impact on larval feeding and weight gain (Table 3). *T. ni* larvae ate significantly more leaf area (*P* < 0.05, *t* test) and gained significantly more weight (*P* < 0.01, *t* test) on plants subjected to fogs of pH 3.0 than on plants exposed to control fogs.

Although a trend for greater average leaf area eaten per larvae was observed for larvae on plants exposed to fogs of pH 2.0 and 2.5, the magnitudes of the differences were less than those at pH 3.0 and were not significant at the *P* < 0.05 level (Table 3). Variation in foliar water content is often an important factor in insect growth and development (Scriber, 1984) but was not significant

and did not provide an explanation for the results in our study (Table 1). Because nitrogen is often a limiting factor for insects (Mattson, 1980), the lack of improvement in weight gain by *T. ni* larvae was surprising, given the elevated percent total nitrogen in plants exposed to 2.0 pH fogs and the increases in soluble proteins and specific free amino acids in plants subjected to 2.5 pH fogs (Tables 1 and 2). However, because nitrogen was added to the system in the form of fertilizer, the nitrogen availability may not have been as limiting in these plants as compared to other systems. Insects feeding on foliage from plants in either acidic fog treatment, therefore, would not have been limited by percent total nitrogen, but the ratios of amino acids conceivably could affect the acceptance or osmotic balance of treated foliage (House and Barlow, 1964; Broadbeck and Strong, 1987).

Several alternative explanations exist for the lack of increased weight gain of *T. ni* larvae on plants with higher percent total nitrogen, soluble protein, or specific free amino acids. One explanation amenable to further testing is that the nitrate form of nitrogen is unsuitable for *T. ni*. A second explanation is based on the observation that test plants from the pH 2.0 and 2.5 fogs had (by subjective evaluation) more necrotic areas, which reasonably could be expected to interfere with palatability or phagostimulation. Large necrotic regions on

TABLE 3. IMPACT OF ACIDIC FOGS ON LARVAL FEEDING, HOST PREFERENCE, LARVAL WEIGHT GAIN, AND PERCENT SURVIVAL OF *T. ni* EGGS

Source	pH of test fog					
	2.0		2.5		3.0	
Eaten on intact plants (cm ²) ^a	<i>df, t value</i>		<i>df, t value</i>		<i>df, t value</i>	
Control plants	8.81	52, -1.043	4.07	52, -1.702 ^b	5.28	55, -2.363*
Test plants	10.16		5.62		8.61	
Eaten in 24-hr leaf choice test (cm ²) ^c	<i>df, t value</i>		<i>df, t value</i>		<i>df, t value</i>	
Control plants	0.66	18, -0.404			1.09	18, -0.388
Test plants	0.73				1.25	
Larval weight gain ^a	<i>df, t value</i>		<i>df, t value</i>		<i>df, t value</i>	
Control plants	0.030	49, 1.503	0.030	42, 0.403	0.019	25, 4.323**
Test plants	0.037		0.028		0.093	
Survival of eggs (%) ^a	<i>df, t value</i>		<i>df, t value</i>		<i>df, t value</i>	
Control plants	83.33	10, 1.209			85.33	10, 1.785
Test plants	75.83				75.83	

^aAnalyzed with an unpaired *t* test; *significance at the $P < 0.05$ level; ** $P < 0.01$.

^bSignificant at $P < 0.1$ level.

^cAnalyzed with a paired *t* test.

Phaseolus species caused by acidic fogs typically have no intact cells and the cellular material coagulates into an undifferentiated mass less than one third the volume of undamaged tissue (Evans et al., 1977; Trumble and Walker, unpublished). Thus, larvae on these plants would spend more time in transit between acceptable or palatable regions and less time feeding than larvae on foliage with minimal damage. A third explanation is that the changes in nitrogen content and quality were at least partially offset by increases in other compounds (phenolics, phytoalexins, etc.) produced in response to damage that deterred feeding and/or assimilation by the larvae. Finally, because *T. ni* is a generalist feeder, the variation in nitrogen form and content between stressed and unstressed plants within the species observed in this study may be small relative to variation among species within the insect's broad host range.

Long-term effects of acidic fogs on the larval population dynamics of insects would be dependent, among other things, on the time following acidic fog exposure, the duration and acidity of the fog, and the rate at which nitrate nitrogen was converted to insect usable or acceptable forms. In our study, no significant ($P < 0.1$) effects of fogs were seen on the survivorship of the egg stage (Table 3) or on survivorship of larvae in any treatment-control comparison from the larval weight gain study or the leaf area consumption study (t test, P was always greater than 0.2). However, the survivorship of larvae was not followed through to pupation, nor were any direct effects of acidic fogs on adult oviposition or larval behavior monitored. Thus, additional research must be conducted before the impact of acidic fogs on the life history or population dynamics of *T. ni* can be estimated with confidence.

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Book Review

CRC Handbook of Natural Pesticides: Methods, Vol. I, Theory, Practice, and Detection.

N. Bushan Mandava (ed.).

Boca Raton, Florida, CRC Press, 1985, Second Printing 1988. 534 pp.

This book is a treasure that should be added to the library of every policy maker, administrator, instructor, and researcher concerned with the use of natural and synthetic chemicals for the protection of crops and regulation of their growth and development.

The book begins with a masterful chapter by the distinguished professor, synthesizer, and policy analyst, David Pimentel. Pimentel summarizes the losses caused by insects, diseases, and weeds, and explains factors and trends in losses and approaches to mitigating them from an ecological perspective. He also includes a tabulation of the environmental and social costs of pesticide use in the United States. A defect in this chapter is Pimentel's failure to use current information on pesticide usage. Thus, Pimentel implies that the volume of insecticide usage continues to increase and that the percent of pesticides used is 60% for herbicides; 25% for insecticides, and 15% for fungicides. Actually, the volume of insecticide usage peaked in 1978, whereas use of herbicides has continued to progressively increase at more than 6% per year and has more than doubled between 1975 and 1985 (see Carlson, 1989; Ayers, 1985; Gianessi, 1987). Thus, currently the percent of total usage of pesticides in the United States is roughly 70% for herbicides, 25% for insecticides and nematocides, and 5% for fungicides.

Chapter 2 on integrated crop management systems by El-Zik and Frisbie is crammed with wonderful facts and concepts from the entire historical record of agriculture. The chapter provides a very comprehensive discussion of cotton physiology in relation to all of the organisms and environmental factors that affect the crop in a negative or positive manner. This chapter should be required reading, not only for students of the cotton plant and its production and protection, but also for all students in agronomy and plant protection. They present the ancient wisdom of Epictetus that "one must not tie his ship to a single anchor, nor life to a single hope."

Chapter 3, by the late Warren C. Shaw, addresses technology for integrated weed management systems. Shaw describes the program classification for integrated pest management that was devised by the now defunct Science

and Education Administration of the U.S. Department of Agriculture. He explains our problems with weeds in relation to the natural successions of plants that occur in disturbed habitats. Agriculture often attempts to reverse these natural successions, and this can be achieved only by applying mechanical or chemical energy to the agroecosystem. Shaw enunciates a clear vision of reducing losses caused by weeds and the cost of their control, and leaves the reader with a sense of mission.

Chapter 4, by Tummala and Gage, is an excellent introduction to the use of computers and models in pest management. The chapter is based on seminal experience of the Michigan State University program.

Chapter 5, by Einhellig, discusses the fascinating phenomenon of allelopathy and supports the general discussion with useful case studies and a discourse on the chemistry of natural products exuded by plants to ward off competitors. The chapter amply demonstrates that the inhibitory as well as stimulating allelopathic capabilities provide a significant but complex channel for improving agricultural production. The chapter should be read by those concerned with groundwater contamination by synthetic organic herbicides.

Chapter 6, by Dickens and Payne, is a clearly written exposition on the fundamental aspects of insect chemoreception and of insect behavior in relation to chemical messengers. The authors have succeeded in distilling the essence of this vast topic into a few pages of considerable pedagogical and heuristic value.

Chapter 7, by Hodosh, Keough, and Luthra, provides a very clear and thorough exposition of the Environmental Protection Agency's requirements for registering biochemical and microbial pesticides. These requirements have continued to evolve, and the reader is advised to contact the agency for current and prospective requirements.

In chapter 8, Upholt compares and contrasts approaches to the regulation of pesticides used by various countries. The chapter is helpful in gaining an understanding of history and principles of pesticide regulation.

In chapter 9, Perkins attempts to apply to applied entomology Kuhr's concept that scientific revolutions occur when one fundamental paradigm is replaced by another. Perkins attempts to apply this concept not to the science of entomology but, rather, to the application of technologies to meet the problems created for man by insects. Thus, Perkins attempts to divide applied entomologists into one school that adheres to the integrated pest management paradigm and another that adheres to the total population management paradigm.

Chapter 10, by Yopp, addresses bioassays for plant hormones and other naturally occurring plant growth regulators. This 148-page compendium of bioassays should be on the desk of every scientist engaged in research on plant hormones growth regulators.

Chapter 11, by Redfern, is a very brief introduction to bioassays of insect

hormones, attractants, repellents, and antifeedants. It provides specific information on the methods used successfully by the author for several decades. Chapter 12, by Reichelderfer, is a very practical guide to assaying the toxic effects on various insects of bacteria, viruses, fungi, and protozoa. These two chapters could be most useful in instructing students and technicians.

This volume is highly recommended to those who set policies and design programs in plant protection, and to scientists and technicians who may benefit from inspiration and practical methods for contributing to this essential endeavor.

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Book Review

CRC Handbook of Natural Pesticides: Methods, Vol. II, Isolation and Identification.

N. Bushan Mandava (ed.).

Boca Raton, Florida, CRC Press, 1985, £776, 545 pp.

The application of natural products plays an increasingly important role in integrated pest management. High selectivity and easy biodegradation render many of them superior to the "classical" synthetic pesticides. N. Bhushan Mandava, as editor-in-chief of the series, has undertaken to integrate the extensive themes in this area. The second volume of the first part, "Methods" (of a series of seven books) deals with general aspects of analytical organic chemistry with some special emphasis on selected compounds. The term "natural pesticides" is generously treated. It is debatable whether biologically active natural products or their derivatives "once . . . applied by external means . . . come under the label 'pesticides' because they contaminate the environment" (Introduction), or whether "pheromones, insect hormones and plant growth regulators are considered together because of their mode of action as pesticides" (p. 452). The term "natural" is also not taken too seriously, since GC-FTIR data of Mirex and its derivatives as well as GC-MS data of Arochlor are included.

The book is divided into two main topics: "Methods for Isolation" (four chapters) and "Methods for Identification" (14 chapters). Basic principles used in the analysis of natural products are compiled, and the nonspecialist who wants a rapid overview is carefully informed about the theoretical background of separation techniques and spectroscopic methods; the expert will also benefit from collected data. Subdirectories for each chapter would have been helpful.

The first part, isolation (30% of the book), starts with a nice and highly condensed review by E.D. Morgan and I.D. Wilson of a broad spectrum of isolation techniques of volatile and nonvolatile organic compounds from complex mixtures. Unfortunately, there are many misdrawings in the chemical formulas which may confuse the non-insider. The next chapter, by G.I. Kingston and M.M. Rao, deals with chromatography and is more oriented to theoretical and technical background than towards practical application. Modern injection techniques in GC, micromethods in derivatization and preparative isolation, as well as multidimensional separation in GC and HPLC are not discussed. Separation of enantiomers on optically active stationary phases, which could easily fill a chapter of its own, is not mentioned. Supercritical fluid chromatography (SFC) may be too new to be included. The next two chapters, written by W.D.

Conway, on countercurrent chromatography and distribution are highly instructive both from the theoretical and practical point of view. Several tables show solvent systems for the separation of various classes of compounds.

After a general introduction, the second part (identification) starts with extensive chapters on UV and IR spectroscopy to which the editor himself contributed. Many tables of absorption bands of functional groups are given. This part might have profited from condensation but nevertheless contains much useful information. Spectra of compounds, which are targets of this book, are presented in three short chapters. A very important contribution on NMR spectroscopy by D. Weisleder perfectly treated the theoretical background and provides extensive information on cephalotaxus alkaloids, rotenoids, phytoecdysones, and gibberellins. It is a pity that modern two-dimensional techniques are omitted. J.M. Ruth wrote an impressive chapter on mass spectrometry, giving a comprehensive overview of theory and application. More spectra of "natural pesticides" would have been highly desirable. Spectra of chlorinated dimethanonaphthalenes and methanoindene derivatives, such as aldrin or heptachlor, respectively, as well as the complete high-resolution MS of the bark beetle pheromone, lineatin (which covers 1% of the book), are certainly for specialists only. Two informative chapters on optical rotatory dispersion/circular dichroism (J.D. Weber) and X-ray diffraction analysis (J.L. Flippen-Anderson, R. Gilardi) deal with the determination of the absolute configuration of compounds. Application to structural assignment of some naturally occurring pesticides are discussed.

The book finishes with three chapters on methods and techniques that enable workers to investigate the effects of certain compounds on target organisms: electron spin resonance (ESR, by E.C. Toolson); fluorescence, including a valuable list of fluorescent derivatives (R.J. Argauer); and an exciting laboratory manual on the methodology for gene expression (G.R. Chandra, G.P. Albaugh, S.M. Krishnan). An additional chapter on radiolabeling techniques would have been nice to have in this context.

Despite these criticisms, the book is a useful collection of topics in the analytical chemistry of natural products, presented by a team of distinguished scientists. Interdisciplinary groups who are interested in a better understanding of analytical chemistry (and the people who are working in this area) should buy it. Many cross references to monographs, reviews, and original papers will help the reader to become an expert.

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Book Review

CRC Handbook of Natural Pesticides, Vol. III, Insect Growth Regulators, Part A and Part B.

E. David Morgan and N. Bushan Mandava (eds.).
Boca Raton, Florida, CRC Press, 1987. 378 pp.

The first chapter is a short introduction to the insect neuroendocrine system by G. Richards and J.A. Hoffman. It is a useful, simple overview of hormones influencing growth and development in insects and the points at which research has, or may in the future, provide means of controlling pest species. I.D. Wilson gives a comprehensive account of known ecdysteroids and their chemical properties (up to 1984), although there are some unclear or slightly erroneous biological points (e.g., supermolts for supernumerary molts). N. Wakabayashi and R.M. Waters deal with juvenile hormones and related compounds. This chapter is a remarkable collation of the literature (875 references), much of it in table form, but it does suffer from being cryptic or confused. There is no attempt to resolve contradictions in the literature and no value judgments to help the reader with inconsistencies listed. There are a number of apparent errors (for example, precocenes are held to act on the brain, although Pratt and others have clearly shown direct effects on the corpora allata). In addition, the many tables telling us that there is an effect without any idea of what the effect is, are of limited value. Furthermore, two references that were checked referred to papers without the supposed data. Part A is completed by a chapter on chemistry and biology of selected insect peptides by W. Mordue and P.J. Morgan. Unfortunately this deals with the topic, as the title suggests, in a manner that is very selective. For example, there has been more work on insulin and glucagon-like hormones by Moreau and others. Overall, however, it is a useful review of chemistry and biology of peptide hormones up to 1985.

Part B deals with ways of influencing growth and development by using naturally occurring chemicals that in some way interfere with insect physiology. The effects described include effects on hormone production or target tissues, as well as more directly by toxic effects, while in some cases modes of action are quite unknown. The first chapter, by A.B. Bořkovec, deals with chemosterilants. These are divided broadly into mutagens and nonmutagens, although unequivocal designation is often not possible. Listed among mutagens are various alkaloids. Among nonmutagens, nutritional deficiencies are mentioned as well as phytecdysteroids and juvenile hormone analogs. A listing of plant-derived chemicals is given, but the chapter is too brief to be particularly useful.

J.C. Reese and C.W. Holyoke review plant secondary compounds (allelochemicals) affecting insect growth and development. After an introduction, the chapter is in the form of tables and chemical structures. For example, work on nonprotein amino acids (called amino acid analogs) and their effects are listed as well as the chemical data for the compounds, and their structures are shown. Similar data are given for phenylpropane derivatives, terpenoids, alkaloids, flavanoids, etc. Although such a compilation cannot be comprehensive and includes results that might have been due to antifeedant activity, it is a useful reference guide. The same authors have a similarly compiled chapter on acute insect toxicants (i.e., causing mortality) from plants. A number of small errors were noted (e.g., spelling of insect names) as well as some major ones (e.g., calling lignans lignins gives cause for concern about chemical expertise).

Finally, there is a chapter entirely devoted to chemicals from neem and chinaberry trees by H. Schmutterer. Genuine growth-disrupting effects are quite fully reviewed, and the current and future uses of the chemicals are discussed.

In summary, the two books provide 378 pages of useful reviews, although the chapters are of very mixed style and quality. Part A mostly does not fit under the overall title of "natural pesticides," and it is a pity that there were not some more detailed articles on actual modes of action. There would have been more to say on antihormones, for example. Libraries will need to have this book even if individuals do not find it worth the money.

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Book Review

CRC Handbook of Natural Pesticides, Vol. IV, Pheromones.

E. David Morgan and N. Bhushan Mandava (eds.).

Boca Raton, Florida: CRC Press, 1988. 494 pp.

A CRC Handbook on the topic of pheromones does not conjure up images of an exciting treatise about new uses of pheromones or new insights into pheromone chemistry, or even a refreshing synthesis of existing ideas. Instead, the prospective reader envisions a rather dry book brimming with facts, figures, and endless tables. This two-book series (Parts A and B) harbors no surprises. In general, the appeal and utility of handbooks strongly depend on the type, the thoroughness, the arrangement, and the presentation of their important data. Thus, the interest in these books will not depend on whether they are interesting, stimulating, or controversial because, as is well known, handbooks rarely are; rather, interest will depend on whether these books are useful, well organized, and complete.

E. David Morgan and N. Bhushan Mandava are the editors of a multivolume series entitled *Handbook of Natural Pesticides*. Volume IV of this series is simply entitled *Pheromones* and consists of two books, Part A and Part B. Even though a handbook about pheromones is timely and potentially useful, the very title of these books strikes a terrible blow to the "cause" of pheromones because it places them within a larger series concerned with "natural pesticides." This designation as a pesticide is clearly misleading (to people outside the field) and, furthermore, it will probably be used to fuel arguments in support of EPA's classification of pheromones as "pesticides." Arguably, no other factor looms larger to people or organizations who want to commercialize the use of (a) pheromone(s) than the ruling by the EPA that a pheromone will be treated the same way as "hard" pesticides. The subject of pheromones would probably have been better served if the CRC organization had treated pheromones as a distinct entity separate from so-called natural pesticides.

However, the above complaints are minor and reflect personal biases; let us now consider the contents of these books. There are six distinct and almost disjointed chapters; five of the six chapters were written by different authors. Curiously, none of the chapters were written by the editors. The mere fact that there are so many different authors tremendously detracts from the book. For example, five of the six chapters are concerned in some way with cataloging actual pheromone substances and therefore each contains structural, analytical,

and biological activity data. The user should beware, however, because each author presents data in widely differing formats.

The first chapter, "Insect Olfaction and Molecular Structure," written by Richard P. Evershed, is a general review of insect olfaction, and in it are sections on the mechanisms of olfaction, the stereochemistry of olfaction, and the termination of the olfaction process by pheromone-degrading enzymes. This chapter is clearly intended to serve as an introduction to the remaining chapters; the reader, however, is left with the feeling that this chapter is somehow out of place in a handbook about pheromones. Although the chapter is well written and reviews a wealth of literature (178 references), it just does not contain the lasting factual information that makes handbooks useful.

Chapters 2–6 contain information about pheromones of the Lepidoptera; Coleoptera; Diptera; Hymenoptera and Isoptera; and Hemiptera, Blattoidea, and Orthoptera, respectively. Each of these chapters contains some type of background information, usually about the discovery and importance of pheromones in each of the orders covered. The individual chapters also contain, more or less, a review of the isolation, identification, and use of pheromones (and other attractants) for control purposes. None of these written reviews represent worthwhile additions to the already large review literature about pheromones.

Conversely, the abundance of factual data found in Chapters 2–6 represents the true merit and lasting value of these books. For instance, Chapter 2, by Yoshio Tamaki, contains information about and is entitled "Pheromones of the Lepidoptera." Table 1 of this chapter lists information on 160 species of Lepidoptera and lists 99 different compounds associated with these species. Table 2 follows with structural characteristics of the different lepidopteran families. In Table 3, the reader finds a list of synthetic sex attractants for male Lepidoptera as determined by field tests. The data in this table represent useful information for field work and pest control; the data do not, however, have any direct connection with pheromones. The chapter is closed with sections on biosynthesis, secretion of pheromones, and utilization of lepidopteran pheromones (and attractants) for pest management. A discussion of pheromone utilization for pest management is a common theme that is found in all remaining chapters with the notable exception of Chapter 5.

The remaining chapters (3–6) follow a similar pattern, although the specifics vary considerably. Chapter 3, "Pheromones of the Coleoptera," by Hans Jurgen Bestmann and Otto Vostrowsky, is probably the most complete chapter. The authors review not only the isolation and identification of coleopteran pheromones, but also their synthesis. The various tables and sections catalog chemical names, structural formulae, synthesis, occurrence of compounds, physical

and analytical properties, spectral data, function, and biological activity information.

The prose sections include discussions of specificity of beetle pheromones (stereobiology), biosynthesis, plant protection by pheromones, monitoring with pheromones, mass trapping, population suppression, disruption of mating, control of forest and shade-tree pests, control of field-crop insect pests, control of stored-product insect pests, control of grassland and pasture insect pests, and formulations of pheromones. Although the chapter is quite thorough and well written, there is unfortunately no quick or simple method of finding a particular pheromone compound or insect species. However, the compounds are numbered so that when one is found, it is easily cross-referenced to other tables in the chapter.

Chapter 4, "Pheromones of Diptera," and Chapter 6, "Pheromones of Hemiptera, Blattoidea, and Orthoptera," were both written by B.S. Fletcher and T.E. Bellas. The information found in the tables of these chapters is arranged similarly to that in the chapter on Coleoptera. The type of information is also quite similar; the significant addition found in this section is CAS Registry Numbers for each compound. Again, there is an apparent lack of design to the arrangement of compounds in the tables. Both chapters stress the importance of practical and useful aspects of these pheromones.

The remaining section is the chapter by James W. Wheeler and Richard M. Duffield entitled "Pheromones of Hymenoptera and Isoptera." These authors clearly had a different outlook when they wrote this chapter. At first glance, the reader is immediately impressed with the vast compilation of data and how well it is organized. The organization in this chapter deserves special mention: The compounds are arranged in groups by functional groups, and, within each group, by molecular formula. The process of locating specific compounds is quick and simple. Closer inspection reveals, however, that the title of this section should have been "Natural Products of Hymenoptera and Isoptera" instead of "Pheromones of . . ." The vast majority of the compounds compiled in their extensive tables are *not* associated with any particular insect behavior or form of communication. What is nearly always provided instead is the source of the material, e.g., from a certain gland or organ. The problem with this chapter is its lack of useful information on biological activity as well as an almost complete lack of potential uses of these compounds for pest control.

As far as handbooks go, this one is middle of the road. On the plus side, there is no other book or series of books that brings together so much factual information about pheromones with corresponding references to the primary literature. In fact, these books represent a good first-choice reference source for

inquiries ranging from simple factual questions on the one hand, to full literature searches on the other. Presumably, the authors' intentions have been fulfilled. The reader, however, suffers from the disjointed nature of the chapters and from the sometimes disorganized presentation of data. Finally, the potential buyer must question how long the information in these handbooks will remain useful in light of the dynamic and unpredictable nature of the entire field of pheromones.

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STATISTICAL ANALYSIS OF THE JOINT INHIBITORY ACTION OF SIMILAR COMPOUNDS¹

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Abstract—A statistical model is presented that characterizes the joint action of similar compounds when the response variable is continuous. The model allows for antagonistic and synergistic as well as similar joint (additive) action of compounds. It also allows for differential potencies in the compounds. A method of statistical analysis using nonlinear regression analysis is presented along with sample SAS code for carrying it out. An efficient experimental design is given in the form of a set of mixture combinations. Two generalizations of the model are discussed. An example is presented relating the joint action of ferulic and vanillic acids to the growth of cucumber seedlings.

Key Words—Synergy, antagonism, similar joint action, mixture.

INTRODUCTION

The purpose of this article is to present an experimental design, a statistical model, and a method of analysis that can be used for mixture experiments when the response variable is continuous. In particular, the methodology is applicable to experiments in which the action of mixtures of two chemically similar compounds is being evaluated.

The potency of a compound, C_1 , relative to another compound, C_2 , is

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defined as the concentration (or dose) of C_1 that must be applied to experimental material to achieve the same effect as that obtained by the application of a unit concentration (or dose) of C_2 . For the compounds under study, we shall assume that relative potency is constant over the range of concentrations under consideration.

The proposed model presumes that the potency of a mixture of two compounds relative to one of the constituent compounds (with respect to a particular response) is proportional to the effective concentration of the mixture, Z . That is, the magnitude of the response, Y , to such a mixture is predictable from the effective concentration alone. If the effective concentration of the mixture, Z , is a linear (additive) function of the concentrations, X_1 and X_2 , of the constituent compounds, we describe their joint effect on the response as being similar joint action. Alternatively, if the effective concentration of the mixture is a nonadditive function of the concentrations of the constituents, the joint effect of the two acids may be antagonistic or synergistic. Antagonistic action occurs when the effective concentration, Z , increases more slowly with increases in X_1 and X_2 than would be expected under similar joint action; whereas synergistic action occurs when Z increases more rapidly. The concept of similar action was first presented by Bliss (1939) and elaborated by Finney (1952). Mixture models for quantal response data are given in Finney where he discussed the concept. Morse (1978) reviews attempts to define the terms antagonism and synergism. She also examines existing models for describing the joint action of two compounds when no antagonism or synergism exists including the additive dose model (ADM) and the multiplicative survival model (MSM). The models proposed in this paper are generalizations of the additive dose model.

METHODS AND MATERIALS

Models

Effective Concentration Model. Suppose that a mixture of two compounds, C_1 and C_2 , is applied to experimental material. Let (X_1, X_2) denote the application of such a mixture with C_1 at concentration X_1 and C_2 at concentration X_2 . If the action of a mixture (X_1, X_2) on the experimental material is determined solely by the value of the function

$$Z = X_1 + \beta_4 X_2 \quad (1)$$

where β_4 is a constant, then we say that the compounds exhibit similar joint action. The value of Z may be thought of as the effective dose or concentration that results from the application (X_1, X_2) . Thus, if compound C_1 is present alone resulting in $(X_1, 0)$, then $Z = X_1$; if compound C_2 is present alone resulting in $(0, X_2)$ then $Z = \beta_4 X_2$. The value of the parameter β_4 is the potency of

compound C_2 relative to that of C_1 as defined above. When, for example, $\beta_4 > 1$, compound C_2 is more potent than C_1 since the effective dose for $(X, 0)$ is $Z = X$ while that for $(0, X)$ is $Z = \beta_4 X$. Equivalent compounds would have $\beta_4 = 1$.

It would be advantageous to have a more general nonlinear model which relates (X_1, X_2) to effective concentration that includes the model for similar joint action as a special case. One such generalization, given below, is obtained by adding to the similar joint action model a multiple of the geometric mean of its two additive components:

$$Z = X_1 + \beta_4 X_2 + 2\beta_5(\beta_4 X_1 X_2)^{1/2} \quad (2)$$

where β_4 and β_5 are constants. This model has the pleasing property that the effective concentration, Z , is expressed in the same units as that shared by X_1 and X_2 . The β_5 parameter, sometimes referred to as the coefficient of synergy, determines the degree of departure of this model from the similar joint action model. Positive values of this parameter result in the effective concentration being greater than would be predicted by similar joint action and therefore correspond to synergistic action. In the same way, negative values of β_5 correspond to antagonistic action. The permissible range for β_5 is $-1 < \beta_5 < +\infty$ and, as such, the degree of antagonism can be judged on a scale ranging from 0 (no antagonism) to -1 (complete antagonism). A comparable range for synergism is obtained through the expression $\beta_5/(1 + \beta_5)$ which ranges from 0 (no synergism) to 1 (complete synergism) as β_5 ranges from 0 to $+\infty$.

Response Model. The response, Y , of the experimental material to the joint action of the compounds as a function of the effective concentration, Z , is frequently sigmoidal in shape. This is particularly true in cases where the nature of the action of the mixture is inhibitory, as with growth. A versatile model that is adequate for many applications is the three-parameter logistic function.

$$Y = \beta_1 / (1 + \exp\{\beta_3[\ln(Z) - \beta_2]\}), \quad \text{for } Z > 0 \quad (3a)$$

$$= \beta_1, \quad \text{for } Z = 0 \quad (3b)$$

The parameters in the model have the following interpretation:

β_1 is the response under control condition (i.e., $X_1 = X_2 = 0$ implies $Y = \beta_1$); β_2 is the value of $\ln(Z)$ required to achieve 50% inhibition, i.e., values of X_1 and X_2 for which $Z = \exp(\beta_2)$ will yield response $Y = \beta_1/2$; and β_3 is the parameter controlling rate at which the curve descends.

Statistical Analysis

Assume that data are available in the form (X_{i1}, X_{i2}, Y_i) , where X_{ij} is the concentration of the j th compound for the i th observation and Y_i is the value of

the response for the i th observation, $i = 1, 2, \dots, n$ and $j = 1, 2$. Consider the five-parameter nonlinear regression model:

$$Y_i = f(X_{i1}, X_{i2}; \beta) + \epsilon_i \quad (4)$$

i is $1, 2, \dots, n$, where $\beta = (\beta_1, \beta_2, \beta_3, \beta_4, \beta_5)$ denote the parameters; $f(X_1, X_2; \beta)$ is $\beta_1 / (1 + \exp\{\beta_3[\ln(Z) - \beta_2]\})$; Z is $X_1 + \beta_4 X_2 + 2\beta_5 \beta_4 X_1 X_2$ ^{1/2}; and ϵ_i , $i = 1, 2, \dots, n$ are independent random errors each with mean zero and variance σ^2 .

The methods of nonlinear regression analysis (see Gallant, 1975) may be used to find the least-squares estimator of the parameter vector β , i.e., that which minimizes the sum of squares:

$$SS(\beta) = \sum_{i=1}^n [Y_i - f(X_{i1}, X_{i2}; \beta)]^2 \quad (5)$$

and find its variance-covariances matrix. Several statistical software packages are available for doing this. Given the model specification (sometimes to include expressions for the partial derivatives of the model with respect to each parameter), the data and starting values for the parameters, these programs will provide least-squares estimates, $\hat{\beta}_j$, for the parameters, β_j , along with estimates, $\hat{\sigma}_j^2$, of their standard errors, σ_j^2 , and confidence intervals for the true parameters. SAS computer code is given in the Appendix as an illustration of the use of PROC NLIN (see SAS Institute Inc., 1985) for analyzing data using this model.

Starting Values. Suitably chosen starting values for the parameters to be used by the nonlinear regression algorithm can ensure convergence and cut down on the time required to achieve it. For β_1 , pick as a starting value, b_1 , the value of the average response, Y , which corresponds to mixture (0, 0) in the data. For β_2 , first take a guess, a_2 , at the value of X_1 which would yield a response equal to $Y = b_1/2$, then take $b_2 = \ln(a_2)$. Or, more simply, set $b_2 = 0$, which works in most cases. For β_3 , for experiments in which the effect of the compounds on the response is thought to be inhibitory, set $b_3 = 1$. For β_4 and β_5 set $b_4 = 1$ and $b_5 = 0$, respectively.

Testing Hypotheses. From this output we may test relevant hypotheses about the values of the true parameters in the model. For testing the two-sided hypotheses, $H_0: \beta_j = \beta_j^0$ versus $H_1: \beta_j \neq \beta_j^0$, where β_j^0 is given, calculate the value of the statistic:

$$t = (\beta_j - \beta_j^0) / \hat{\sigma}_j \quad (6)$$

and reject the null hypothesis, H_0 , in favor of the alternative, H_1 , whenever $|t| > z_{1-\alpha/2}$, where if Z is a standard normal random variable, z_α satisfies $P(Z \leq z_\alpha) = \alpha$ (e.g., $z_{0.975} = 1.96$). For one sided hypotheses, $H_0: \beta_j \geq \beta_j^0$ versus $H_1: \beta_j < \beta_j^0$, reject H_0 whenever $t < -z_{1-\alpha}$ and for $H_0: \beta_j \leq \beta_j^0$ versus $H_1: \beta_j > \beta_j^0$, reject H_0 whenever $t > z_{1-\alpha}$.

Hypotheses of Interest. To determine whether the experimental material exhibits a significant dose-response to the mixtures, we can test the null hypothesis $H_0: \beta_3 = 0$ versus the alternative hypothesis $H_1: \beta_3 \neq 0$, that is set $\beta_3^0 = 0$. This can be tested by the two-sided test described above or by referring to the confidence interval for β_3 and rejecting H_0 whenever the interval fails to cover zero. If this hypothesis cannot be rejected, then the analysis should not be continued since the estimates obtained for the other parameters would be meaningless.

The hypothesis of similar joint action (lack of synergism or antagonism) corresponds to $H_0: \beta_5 = 0$ versus $H_1: \beta_5 \neq 0$ and can be tested using the one-sided test or by noting whether or not the value zero is included in the confidence interval. Alternatively, if the purpose of the experiment is to prove synergism, then we wish to test $H_0: \beta_5 \leq 0$ versus $H_1: \beta_5 > 0$ which can be tested using the one-sided test. A test for antagonism may be similarly defined.

The hypothesis of equally potent compounds is expressed as $H_0: \beta_4 = 1$ versus $H_1: \beta_4 \neq 1$ and can be tested using the two-sided test or by noting whether the value one is or is not included in the confidence interval for β_4 .

Blocked or Replicated Experiments: The model can be generalized to allow experiments to be run in r replicates or blocks. For this purpose, the parameter β_1 is permitted to assume a different value in each replicate: $\beta_{11}, \beta_{12}, \dots, \beta_{1r}$. The data become $(X_{i1}, X_{i2}, L_{i1}, L_{i2}, \dots, L_{ir}, Y_i)$, where $L_{ik} = 1$ when the i th observation is from the k th replicate and zero otherwise, where $i = 1, 2, \dots, n$ and $k = 1, 2, \dots, r$. Then the response model becomes:

$$Y = \beta_{1.} / (1 + \exp\{\beta_3[\ln(Z) - \beta_2]\}), \quad \text{for } Z > 0 \quad (7a)$$

$$= \beta_{1.}, \quad \text{for } Z = 0 \quad (7b)$$

where $\beta_{1.} = L_1\beta_{11} + L_2\beta_{12} + \dots + L_r\beta_{1r}$. Starting value, b_{1k} , for β_{1k} may be taken as the value of the response to the (0, 0) mixture in the k th replicate, $k = 1, 2, \dots, r$. The tests of hypothesis are carried out as before.

RESULTS AND DISCUSSION

Experimental Design

The proposed experimental design was devised to be efficient (in the sense of D-optimality) for situations where β_1 is arbitrary, β_3 is in the range 1 to 3, and β_5 ranges from extremely antagonistic ($\beta_5 = -0.75$) to extremely synergistic ($\beta_5 = 3$). This covers a large percentage of cases likely to be encountered in practice. Since the design may vary greatly with changes in the parameters $\exp(\beta_2) = \alpha_2$ (= concentration required to achieve 50% inhibition) and β_4 ,

estimates or guesses at their values must be supplied. Lacking all knowledge of β_4 , set it equal to 1.

The following seven-point treatment design should be run in three or more replicates. Assume that by prior experimentation or other means preliminary estimates, a_2 and b_4 , are available for the parameters $\alpha_2 = \exp(\beta_2)$ and β_4 . In each replicate, runs should be made at mixtures ($\mu\text{mol/g}$):

$$(0, 0), (0.5*a_2, 0), (0, 0.5*a_2/b_4), (1.3*a_2, 0), (0, 1.3*a_2/b_4), \\ (0.5*a_2, 0.5*a_2/b_4), (1.4*a_2, 1.4*a_2/b_4)$$

Thus, for example, if $a_2 = 0.2$ and $b_4 = 0.6$, the design becomes $(0, 0)$, $(0.1, 0)$, $(0, 0.17)$, $(0.26, 0)$, $(0, 0.43)$, $(0.1, 0.17)$, $(0.28, 0.47)$. If the compounds are known to be antagonistic, the last mixture may be omitted. If they are known to be synergistic, the next to last may be omitted.

Testing Model Assumptions

The model described depends on the assumption that the relative potency of the two compounds is constant. To test this assumption, we can define a generalization of the effective concentration model

$$Z = X_1 + [\beta_4(1 + X_2)^{\beta_6}X_2] + 2\beta_5[\beta_4(1 + X_2)^{\beta_6}X_1X_2]^{1/2} \quad (8)$$

where β_6 is a new parameter which, by taking values different from zero, can allow for changes in relative potency as a function of concentration. In the same way that equations 1 and 2 were combined to define the nonlinear model (equation 4), equation 8 can be combined with equation 2 to define an extended six-parameter nonlinear model. It can be fit using nonlinear least squares. SAS code is provided in the Appendix for fitting this model using PROC NLIN. In the context of this model, the hypothesis of constant relative potency is $H_0: \beta_6 = 0$ versus $H_1: \beta_6 \neq 0$ and can be tested using the one-sided test described earlier or by noting whether or not the confidence interval includes zero. If the null hypothesis is rejected, then the proposed analysis may be misleading.

Generalizations for Three or More Compounds

The model described can be generalized to three or more compounds. For this purpose, only the model for effective concentration need be extended. While a number of generalizations of the effectiveness model are possible, one obvious choice for allowing three compounds, C_1 , C_2 , and C_3 with concentrations (X_1, X_2, X_3) would be:

$$Z = X_1 + \beta_4X_2 + \beta_5X_3 + \beta_6(\beta_4X_1X_2)^{1/2} + \beta_7(\beta_5X_1X_3)^{1/2} \\ + \beta_8(\beta_4\beta_5X_2X_3)^{1/2} + \beta_9(\beta_4\beta_5X_1X_2X_3)^{1/3}$$

Similar joint action results when $\beta_6 = \beta_7 = \beta_8 = \beta_9 = 0$. The parameters β_4 and β_5 are, respectively, the potencies of compounds C_2 and C_3 relative to C_1 .

Example

Consider data from an experiment to study the joint action of two compounds, ferulic (C_1) and vanillic (C_2) acid, on the growth of cucumber seedlings. The response of interest was the increase in total leaf area over a fixed interval of time (i.e., mean absolute rate of leaf expansion). The experiment was run in $r = 3$ replicates at concentrations (0, 0), (0, 0.25), (0.0.5), (0.25, 0), (0.5, 0), and (0.25, 0.25), where concentrations are expressed in micromoles per gram. The experimental design used in this example was devised before the efficient design presented above was developed and, as such, differs from it. The SAS code and data listed in the appendix were used to obtain the analysis in Table 1.

From Table 1 we note that for the extended (six-parameter) model $H_0: \beta_6 = 0$ versus $H_0: \beta_6 \neq 0$ we get $t = (0.300 - 0)/2.9 = 0.10 < z_{0.995} = 2.58$ and therefore cannot reject $\beta_6 = 0$. We conclude that relative potency is constant.

For the five-parameter model, we first note that β_3 is significantly greater than zero, indicating that inhibition of leaf growth had a dose-response with the compound concentration. We note further that coefficient of synergy, β_5 , is significantly less than zero at the 1% level since

$$t = (-0.413 - 0)/0.092 = -4.49 < -z_{0.99} = -2.33.$$

From this we conclude that $\beta_5 < 0$ so that the action of the two acids is antagonistic. Finally we see that the confidence interval for the relative potency, β_4 , includes only values less than one, leading to the conclusion that with respect to the growth of cucumber plants, vanillic acid is less potent than equal con-

TABLE 1. PARAMETER ESTIMATES, STANDARD ERRORS (SE), AND CONFIDENCE INTERVALS (CI)

Parameter	5-Parameter model				6-Parameter model	
	Estimate	SE	95% CI		Estimates	SE
β_2	-1.64	0.16	-1.98	-1.30	-1.66	0.26
β_3	2.72	0.74	1.10	4.34	2.56	1.7
β_4	0.626	0.107	0.391	0.861	0.564	0.59
β_5	-0.413	0.092	-0.615	-0.212	-0.410	0.11
β_6	0.000				0.300	2.9

centrations of ferulic acid. To confirm this we can test $H_0: \beta_4 \geq 1$ versus $H_1: \beta_4 < 1$. Using the 1% level we compute

$$t = (0.626 - 1)/0.107 = -3.50 < -z_{0.99} = -2.33$$

and conclude that $\beta_4 < 1$.

We can compute estimates of the concentration required to achieve 50% inhibition for ferulic acid as

$$\exp(\beta_2) = \exp(-1.64) = 0.194 \mu\text{mol/g}$$

and for vanillic acid as

$$\exp(\beta_2)/\beta_4 = \exp(-1.64)/0.626 = 0.310 \mu\text{mol/g}.$$

APPENDIX

SAS code for fitting the model and testing hypotheses about the parameters.

```

DATA A;
INPUT Y X1 X2 C;
* Y = RESPONSE VARIABLE;
* X1 AND X2 = CONCENTRATIONS OF THE TWO COMPOUNDS;
* C = BLOCK OR REPLICATE NUMBER - 3 BLOCKS IN EXAMPLE;

C1 = (C = 1); C2 = (C = 2); C3 = (C = 3);
CARDS;
16.4720 0.00 0.00 1
11.3889 0.00 0.00 2
16.7488 0.00 0.00 3
 9.2972 0.00 0.25 1
 9.5356 0.00 0.25 2
10.1047 0.00 0.25 3
 3.8527 0.00 0.50 1
 3.5682 0.00 0.50 2
 2.1378 0.00 0.50 3
 1.8917 0.25 0.00 1
 6.6595 0.25 0.00 2
 6.4365 0.25 0.00 3
 5.1907 0.25 0.25 1
 7.7208 0.25 0.25 2
 3.1990 0.25 0.25 3

```

```
0.8690 0.50 0.00 1
1.7302 0.50 0.00 2
0.9536 0.50 0.00 3
```

```
;
```

```
PROC NLIN;
```

```
* ----- Starting values for parameters ----- ;
```

```
PARMS
```

```
B11 = 16.5 B12 = 11.4 B13 = 16.7
```

```
B2 = 0
```

```
B3 = 1
```

```
B4 = 1
```

```
B5 = 0;
```

```
* ----- Model specification ----- ;
```

```
TOL = 0.0001;
```

```
Z = X1 + B4*X2 + 2*B5*SQRT(B4*X1*X2);
```

```
TO = B11*C1 + B12*C2 + B13*C3;
```

```
IF Z < TOL THEN T1 = 0; ELSE T1 = EXP (B3*(LOG(Z) - B2));
```

```
T2 = 1/(1 + T1);
```

```
T3 = -T0*T2*(1 - T2);
```

```
MODEL Y = T0*T2;
```

```
* -- Partial derivatives of f with respect to each parameter;
```

```
DZDB4 = X2 + B5*SQRT(X1*X2/B4); * Partial of Z wrt B4;
```

```
DZDB5 = 2*SQRT(B4*X1*X2); * Partial of Z wrt B5;
```

```
DER.B11 = C1*T2;
```

```
DER.B12 = C2*T2;
```

```
DER.B13 = C3*T2;
```

```
DER.B2 = T3*(-B3);
```

```
IF Z < TOL THEN DER.B3 = 0; ELSE DER.B3 = T3*(LOG(Z) - B2);
```

```
IF Z < TOL THEN DER.B4 = 0; ELSE DER.B4 = B3*T3*DZDB4/Z;
```

```
IF Z < TOL THEN DER.B5 = 0; ELSE DER.B5 = B3*T3*DZDB5/Z;
```

```
PROC NLIN;
```

```
* ----- Extended nonlinear model ---- ;
```

```
* ----- Starting values for parameters;
```

```
PARMS
```

```
B11 = 16.5 B12 = 11.4 B13 = 16.7
```

```
B2 = 0
```

```
B3 = 1
```

B4 = 1
 B5 = 0
 B6 = 0;

* ---- Model specification ---- ;

TOL = 0.0001;

Z = X1 + B4*((1 + X2)**B6)*X2 + 2*B5*SQRT(B4*
 ((1 + X2)**B6)*X1*X2);

T0 = B11*C1 + B12*C2 + B13*C3;

IF Z < TOL THEN T1 = 0; ELSE T1 = EXP(B3*(LOG(Z) - B2));

T2 = 1/(1 + T1);

T3 = -T0*T2*(1 - T2);

MODEL Y = T0*T2;

* -- Partial derivatives of f with respect to each parameter;

DZDB4 = ((1 + X2)**B6)*X2 + B5*SQRT(X1*((1 + X2)**B6)*X2/B4);

DZDB5 = 2*SQRT(B4*X1*((1 + X2)**B6)*X2);

DZDB6 = B4*((1 + X2)**B6)*X2*LOG(1 + X2) +
 2*B5*SQRT(B4*((1 + X2)**B6)*X1*X2)*LOG(1 + X2);

DER.B11 = C1*T2;

DER.B12 = C2*T2;

DER.B13 = C3*T2;

DER.B2 = T3*(-B3);

IF Z < TOL THEN DER.B3 = 0; ELSE DER.B3 = T3*(LOG(Z) - B2);

IF Z < TOL THEN DER.B4 = 0; ELSE DER.B4 = B3*T3*DZDB4/Z;

IF Z < TOL THEN DER.B5 = 0; ELSE DER.B5 = B3*T3*DZDB5/Z;

IF Z < TOL THEN DER.B6 = 0; ELSE DER.B6 = B3*T3*DZDB6/Z;

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EFFECTS OF MIXTURES OF PHENOLIC ACIDS ON LEAF AREA EXPANSION OF CUCUMBER SEEDLINGS GROWN IN DIFFERENT pH PORTSMOUTH A₁ SOIL MATERIALS¹

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Abstract—Cucumber seedlings growing in A₁-horizon Portsmouth soil material adjusted to pH 5.2, 6.0, or 6.9 were treated with 0, 0.25 or 0.5, $\mu\text{mol/g}$ soil ferulic acid, *p*-coumaric acid, vanillic acid, or an equal mixture (0.5 $\mu\text{mol/g}$ total) of two acids every other day. A total of five treatments was given starting with day 7 from seeding. Absolute rates of leaf expansion were determined for seedlings. The experiment was terminated when seedlings were 17 days old. All three phenolic acids inhibited leaf expansion. The dose required for 50% inhibition of absolute rates of leaf expansion increased as pH of the soil systems increased. The order of toxicity based on 50% dose and relative potency were as follows: ferulic acid > vanillic acid = *p*-coumaric acid. Effects of mixtures of phenolic acids on absolute rates of leaf expansion, when compared to the effects of individual phenolic acids, were found to be antagonistic for the ferulic-vanillic acid mixture and the ferulic-*p*-coumaric acid mixture in the pH 5.2 soil systems. Several phenolic acid treatments were required before antagonistic effects of mixtures were evident. In all other instances, when treatment effects were significant, the effects of individual phenolic acids were additive.

Key Words—Allelopathy, cucumber, *Cucumis sativus*, phenolic acid mixtures, similar joint action analysis, leaf area expansion, pH.

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INTRODUCTION

Mixtures of simple, water-soluble, phytotoxic phenolic acids have been isolated from various soils. The cinnamic acid derivatives, *p*-coumaric acid and ferulic acid, and the benzoic acid derivatives, *p*-hydroxybenzoic acid and vanillic acid, are the most frequently observed in soil extracts, although various other phenolic acids have also been identified (Whitehead 1964; Guenzi and McCalla, 1966; Whitehead et al., 1981, 1983; Kuiters and Denneman, 1987; Kuiters et al., 1987). Estimates of the concentrations of individual phenolic acids that are directly available in soil solution to interact with seeds or roots are frequently below those required for growth inhibition. A number of researchers, however, have suggested that mixtures composed of noninhibitory concentrations of individual phenolic acids could inhibit seed germination or plant growth (see Einhellig, 1987). Predicting how mixtures of varying compositions (i.e., numbers of phenolic acids and their concentrations) may affect seed germination or plant growth in soils is not simple, since effects of individual phenolic acids in mixtures may be synergistic, additive, or antagonistic (Rasmussen and Einhellig, 1977; Blum et al., 1984, 1985a; Einhellig, 1987). Furthermore, it appears that the action of the individual phenolic acids in a mixture, and therefore the action of the mixture, will be directly or indirectly modified by soil pH and microbial activity (Blum et al., 1985a,b; 1987; Shann and Blum, 1987; Blum and Shafer, 1988).

The objective of this study was twofold: (1) to study the impact of mixtures of phenolic acids on seedling growth and (2) to describe how the effects of phenolic acid mixtures on growth may be modified by soil pH.

METHODS AND MATERIALS

General Aspects. Cucumber seeds (*Cucumis sativus* cv Early Green Cluster; Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated in the dark at 28–30°C in trays containing sterile vermiculite and Hoagland's solution (Hoagland and Arnon, 1950). After 48 hr, the trays containing the seedlings were transferred to a light bank and given a 12-hr light period (189 μ Einsteins/m²/sec for 12 hr) and a 12-hr dark period. Seedlings were planted into 155-ml cups (one per cup) containing 150 g of a Portsmouth A₁ soil-sand mixture (see below). Cups did not have drainage holes. The cups were inserted through holes in a plywood sheet so that everything below the soil surface was protected from direct irradiance of the light banks (Blum and Dalton, 1985). Seedlings were grown at room temperature (21–30°C) with a 12-hr light period. Seedlings were supplied with 7 ml double-strength Hoagland's solution every other day. Sufficient distilled water was added daily to bring the weight of any given cup (160 g for cup and soil) and its seedling to approximately 190 g.

Cups containing the seedlings were not allowed to go below 170 g. For further details about the cup system, see Blum et al. (1987). Length and width measurements were taken for each leaf (excluding cotyledons) every other day.

Substrate and pH Treatments. A₁-horizon Portsmouth (fine loamy, mixed, thermic Typic Umbraqualts) soil material was obtained from the Coastal Plain of North Carolina. General soil characteristics were described by Blum et al. (1987). Aluminum sulfate or calcium hydroxide (to adjust the pH of the soil material) and Ottawa quartz sand (2 sand-1 soil by weight) were added, and the resulting soil preparation was then mixed for 12-15 hr in a twin-shelled mixer. Soil pH values (based on a 1:1 soil-water mixture) were adjusted to 5.2, 6.0, and 6.9.

Phenolic Acid Treatments. Seedlings were treated every other day (maximum of five times) with ferulic, vanillic, or *p*-coumaric acid (0, 0.25, or 0.5 $\mu\text{mol/g}$ soil material) or equal molar mixtures (0.25 $\mu\text{mol/g}$ each) of two phenolic acids starting with day 7. Phenolic acid treatments were alternated with nutrient solution applications. Solution pH values were adjusted to soil pH values with NaOH.

Experimental Design. The experimental design for the seedling bioassay was as follows: three pH levels, 10 phenolic acid combinations (including control) and three replicates. Replication was in complete blocks, a complete set of treatments being randomly arranged under each of three lightbanks.

Data Analyses. Leaf area was determined from length and width measurements of leaves and the following equation: Leaf area = $-1.457 + 0.00769(L \times W)$, $P = 0.0001$, $r^2 = 0.98$, $N = 121$ where leaf area is in cm^2 and length (L) and width (W) values are in mm (Blum and Dalton, 1985). Mean absolute rates of leaf expansion (AGR) were calculated for each seedling by the equation: $\text{AGR} = \text{leaf area of seedling at time}_{x+1} - \text{leaf area of seedling at time}_x$ (Radford, 1967). Since seedling measurements were taken at two-day intervals, values are based on two-day periods. Data were analyzed by analysis of variance or nonlinear regressions and joint action analysis (Gerig et al., 1989).

RESULTS

Means for absolute rates of leaf expansion (AGR) used in the similar joint action analysis are presented in Table 1. No significant phenolic acid treatment effects [i.e., the slopes, (β_3) were not significantly different from zero] were noted for the pH 6.9 soil system, thus model parameters are presented only for the pH 5.2 and 6.0 soil systems (Tables 2-7).

As pH of the soil systems increased, AGR increased (see Table 1 and β_1 values in Tables 2-7). The number of phenolic acid treatments required before β_3 (rate parameter, slope) was significantly different from zero increased with increasing pH of the soil systems.

TABLE 1. MEANS ($N = 3$) FOR ABSOLUTE RATES OF LEAF EXPANSION (AGR) FOR CUCUMBER SEEDLINGS GIVEN MULTIPLE TREATMENTS OF FERULIC (FER), *p*-COUMARIC (PCO), AND VANILLIC (VAN) ACIDS INDIVIDUALLY OR IN COMBINATION.

Phenolic acids	Conc ($\mu\text{mol/g}$)	Soil pH	AGR ($\text{cm}^2/2$ days) during growth periods (days)				
			9-11	11-13	13-15	15-17	
FER	0.00	5.2	6.80	9.19	14.87	23.65	
		6.0	8.56	11.26	21.47	29.08	
		6.9	10.67	17.87	30.89	39.08	
	0.25	5.2	5.28	5.78	4.99	5.83	
		6.0	8.06	7.52	10.94	18.05	
		6.9	9.90	15.12	29.41	36.12	
	0.50	5.2	2.39	1.59	1.18	2.99	
		6.0	5.18	5.02	7.02	9.31	
		6.9	7.56	10.86	21.65	30.40	
PCO	0.25	5.2	6.47	5.67	9.70	11.01	
		6.0	7.60	10.64	19.96	21.66	
		6.9	10.62	18.15	31.45	40.31	
	0.50	5.2	4.66	4.26	3.16	6.14	
		6.0	6.64	7.83	8.91	16.08	
		6.9	9.37	15.47	23.25	38.89	
	VAN	0.25	5.2	4.87	5.52	9.64	14.09
			6.0	7.51	9.67	14.90	23.32
			6.9	11.65	18.09	31.25	41.63
0.50		5.2	2.52	3.12	3.19	5.20	
		6.0	4.38	6.90	9.15	13.53	
		6.9	7.95	13.85	23.96	35.90	
FER/PCO		0.25/	5.2	3.10	4.98	4.39	6.58
		0.25	6.0	4.67	4.57	6.91	9.29
		6.9	8.56	14.62	23.26	29.53	
FER/VAN	0.25/	5.2	3.21	4.66	5.37	7.54	
	0.25	6.0	7.14	6.62	9.38	16.43	
	6.9	10.10	14.78	25.81	36.80		
PCO/VAN	0.25/	5.2	3.33	4.20	3.00	4.09	
	0.25	6.0	5.13	6.11	9.02	14.70	
	6.9	10.76	16.78	29.09	41.85		

TABLE 2. SUMMARY OF JOINT ACTION ANALYSIS OF EFFECTS OF FERULIC (FER) AND VANILLIC (VAN) ACID ON ABSOLUTE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS (pH 5.2)

	Growth period (days from seed)			
	9-11	11-13	13-15	15-17
β_1 (control values)	6.70	9.13	14.97	23.80
β_2 (log-dose required for 50% inhibition)	-0.90	-1.23	-1.64	-1.87
FER (values in $\mu\text{mol/g}$)	0.41	0.29	0.19	0.15
VAN	0.40	0.34	0.31	0.29
β_3 (rate parameter; compared to 0)	2.41 ^a	2.25 ^a	2.72 ^a	2.12 ^a
β_4 (relative potency; compared to 1)	1.02	0.86	0.63 ^a	0.53 ^a
β_5 (coefficient of synergy; compared to 0; 0: similar or independent joint action, +: synergistic, -: antagonistic)	-0.19	-0.38 ^a	-0.41 ^a	-0.44 ^a

^aSignificant at 5% level.

TABLE 3. SUMMARY OF JOINT ACTION ANALYSIS OF EFFECTS OF FERULIC (FER) AND VANILLIC (VAN) ACID ON ABSOLUTE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS (pH 6.0)

	Growth period (days from seed)			
	9-11	11-13	13-15	15-17
β_1 (control values)	8.63	11.35	21.53	29.20
β_2 (log-dose required for 50% inhibition)	-0.54	-0.88	-1.31	-1.11
FER (values in $\mu\text{mol/g}$)	0.58	0.42	0.27	0.33
VAN	0.50	0.71	0.42	0.48
β_3 (rate parameter; compared to 0)	2.79	1.48 ^a	1.34 ^a	1.98 ^a
β_4 (relative potency; compared to 1)	1.16	0.59	0.64	0.69 ^a
β_5 (coefficient of synergy; compared to 0; 0: similar or independent joint action, +: synergistic, -: antagonistic)	-0.39	-0.17	-0.20	-0.32

^aSignificant at 5% level.

TABLE 4. SUMMARY OF JOINT ACTION ANALYSIS OF EFFECTS OF FERULIC (FER) AND *p*-COUMARIC (PCO) ACIDS ON ABSOLUTE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS (pH 5.2)

	Growth period (days from seed)			
	9-11	11-13	13-15	15-17
β_1 (control values)	6.86	9.06	14.82	23.62
β_2 (log-dose required for 50% inhibition)	-0.94	-1.27	-1.66	-2.24
FER (values in $\mu\text{mol/g}$)	0.39	0.28	0.19	0.11
PCO	0.68	0.40	0.32	0.23
β_3 (rate parameter; compared to 0)	2.64 ^a	1.81 ^a	2.77 ^a	1.31 ^a
β_4 (relative potency; compared to 1)	0.57 ^a	0.70	0.60 ^a	0.47 ^a
β_5 (coefficient of synergy; compared to 0; 0: similar or independent joint action, +: synergistic, -: antagonistic)	-0.08	-0.44 ^a	-0.37 ^a	-0.44 ^a

^aSignificant at 5% level.

TABLE 5. SUMMARY OF JOINT ACTION ANALYSIS OF EFFECTS OF FERULIC (FER) AND *p*-COUMARIC (PCO) ACID ON ABSOLUTE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS (pH 6.0)

	Growth period (days from seed)			
	9-11	11-13	13-15	15-17
β_1 (control values)	8.40	11.58	22.24	28.92
β_2 (log-dose required for 50% inhibition)	-0.51	-0.91	-1.27	-1.11
FER (values in $\mu\text{mol/g}$)	0.60	0.40	0.28	0.34
PCO	0.79	0.89	0.47	0.57
β_3 (rate parameter; compared to 0)	2.70	1.54 ^a	2.21 ^a	1.57 ^a
β_4 (relative potency; compared to 1)	0.76	0.45 ^a	0.60 ^a	0.60 ^a
β_5 (coefficient of synergy; compared to 0; 0: similar or independent joint action, +: synergistic, -: antagonistic)	0.19	0.46	0.01	0.35

^aSignificant at 5% level.

TABLE 6. SUMMARY OF JOINT ACTION ANALYSIS OF EFFECTS OF VANILLIC (VAN) AND *p*-COUMARIC (PCO) ACIDS ON ABSOLUTE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS (pH 5.2)

	Growth period (days from seed)			
	9-11	11-13	13-15	15-17
β_1 (control values)	6.87	9.16	14.88	23.74
β_2 (log-dose required for 50% inhibition)	-0.98	-1.12	-1.19	-1.27
VAN (values in $\mu\text{mol/g}$)	0.38	0.33	0.31	0.28
PCO	0.73	0.41	0.32	0.25
β_3 (rate parameter; compared to 0)	2.28 ^a	1.20	2.78 ^a	1.87 ^a
β_4 (relative potency; compared to 1)	0.52 ^a	0.80	0.96	1.13
β_5 (coefficient of synergy; compared to 0; 0: similar or independent joint action, +: synergistic, -: antagonistic)	0.02	-0.18	-0.005	0.21

^aSignificant at 5% level.

TABLE 7. SUMMARY OF JOINT ACTION ANALYSIS OF EFFECTS OF VANILLIC (VAN) AND *p*-COUMARIC (PCO) ACID ON ABSOLUTE RATES OF LEAF EXPANSION OF CUCUMBER SEEDLINGS (pH 6.0)

	Growth period (days from seed)			
	9-11	11-13	13-15	15-17
β_1 (control values)	8.40	11.31	21.71	29.01
β_2 (log-dose required for 50% inhibition)	-0.63	-0.50	-0.91	-0.71
VAN (values in $\mu\text{mol/g}$)	0.53	0.61	0.40	0.49
PCO	0.83	0.75	0.47	0.53
β_3 (rate parameter; compared to 0)	2.44	2.14	2.48 ^a	1.75 ^a
β_4 (relative potency; compared to 1)	0.64	0.81	0.85	0.93
β_5 (coefficient of synergy; compared to 0; 0: similar or independent joint action, +: synergistic, -: antagonistic)	0.09	-0.24	0.01	-0.005

^aSignificant at 5% level.

The dose [$\mu\text{mol/g}$; $\alpha_2 = \exp(\beta_2)$] required for 50% inhibition of AGR increased as pH of the soil systems increased. The dose for 50% inhibition was $48 \pm 8\%$ ($\bar{X} \pm \text{SE}$) greater for the pH 6.0 than for the pH 5.2 systems. The dose required for 50% inhibition decreased with additional phenolic acid treatments. The dose of phenolic acid required for 50% inhibition of AGR for the 15 to 17-day growth period was $39 \pm 6\%$ lower than for the 9 to 11-day growth period. The order of toxicity of the phenolic acids based on the 50% dose and relative potency (β_4) was as follows: ferulic acid > vanillic acid = *p*-coumaric acid.

Effects of mixtures of phenolic acids on AGR when compared to the effects of individual phenolic acids were found to be antagonistic [i.e., the coefficients of synergy (β_5) were significantly different from zero and negative] for the ferulic-vanillic acid mixture and the ferulic-*p*-coumaric acid mixture in the pH 5.2 soil systems. The joint action of two compounds is antagonistic if, when applied together, they decrease each other's effectiveness. Several phenolic acid treatments were required before antagonistic effects of mixtures on AGR were evident. In all other instances where β_3 values were significantly different from zero (i.e., significant phenolic acid effects), the effects of the mixtures, compared to the effects of the individual phenolic acids, were additive (similar joint action; β_5 was not significantly different from zero).

DISCUSSION

A pH range of 6–6.5 is recommended for best growth of cucumber plants (Hughes et al., 1983). The pH treatments in this study were 5.2, 6.0, and 6.9. No phenolic acid effects were observed at pH 6.9. Maximum effects of phenolic acids were observed at pH 5.2, a pH below the optimum pH range for cucumber seedling growth, but a pH that is often found in field soils. The greater inhibition associated with the pH 5.2 soil systems could have been a result of greater sensitivity of cucumber seedlings to phenolic acids under the suboptimal growth conditions of the pH 5.2 soils. It is more likely, however, that the main reason for these differences in response is related to the solubility and ionization of the phenolic acids. The pK_a values for ferulic, vanillic, and *p*-coumaric acid fall within the 4.5–5.0 range. Solubility and ionization (phenolic acids become negatively charged) of these phenolic acids increase as the pH of the soil solution increases. Harper and Balke (1981) concluded from their studies with salicylic acid ($\text{pK}_a = 3$) and oat root tissue that membranes were more permeable to the undissociated form of salicylic acid found under acidic conditions. This also appeared to be the case for the uptake of ferulic and *p*-hydroxybenzoic acid by cucumber seedlings in nutrient culture (Shann and Blum, 1987). In addition,

the recovery of exogenously applied phenolic acids from sterile acidic Portsmouth soil has been found to be greater than from sterile neutral Portsmouth soil, suggesting that more phenolic acid molecules may be available for root uptake at the lower pH (Dalton et al., 1983). Under acidic conditions, a residual carry-over of phenolic acid from one treatment period to another is also more likely to occur than under more neutral conditions (Blum et al., 1987).

These observations may explain why, under acidic conditions, fewer phenolic acid treatments were required for initial inhibition of AGR and lower concentrations were required for 50% inhibition of AGR. Residual carry-over could also explain why, under acidic conditions, the 50% inhibition concentration declined with increasing number of phenolic acid treatments. Data from nutrient culture studies and soil studies (unpublished data) suggested that cucumber seedlings were not more susceptible or tolerant to ferulic acid exposures after preconditioning with ferulic acids (Blum and Dalton, 1985). Growth, in fact, recovered very rapidly once phenolic acid was removed from the root environment (Blum and Rebbeck, 1988). We suspect, however, that the role of residual carry-over was minimal for these experiments, since recovery of ferulic acid from nonsterile Portsmouth A₁ horizon soil was essentially zero 24 hr after ferulic acid treatment (Blum et al., 1987; unpublished data).

Another potential reason for the decline of the 50% inhibition concentration with time may have been the salinization of the soil systems (no drainage holes) by the additions of double-strength Hoagland's solution. The fact that the number of milliliters of double-strength Hoagland's solution required for best growth increased with age of the seedlings and that growth inhibition required applications of 9 ml or more suggested that this was not the case (Blum et al., 1987). The major reason for the decline of the 50% inhibition concentration with time we suspect was the continued suppression of AGR by the multiple ferulic acid treatments while AGR continued unchecked for the control seedlings.

Antagonistic effects of individual phenolic acids in a mixture may not be directly related to the total concentration of a mixture, but more likely to the magnitude of effects for each phenolic acid in the mixture (Blum et al., 1985a). The data here support this observation, since antagonistic effects were observed only under acidic conditions, conditions of maximum growth inhibition. Furthermore, antagonistic effects were only noted when ferulic acid was present in the mixture. Shann and Blum (1987) noted that ferulic acid inhibited the uptake of *p*-hydroxybenzoic acid by cucumber roots, but that *p*-hydroxybenzoic acid did not affect the uptake of ferulic acid. It is not clear why this occurs, but a similar inhibition of ferulic acid of uptake of other phenolic acids is possible, particularly under acidic conditions where maximum uptake rates occur.

The persistent question in the area of allelopathic interactions has been

whether available concentrations of inhibitors in soil environments are adequate to inhibit plant growth. Estimates of concentrations of individual phenolic acids in soils that are directly available to interact with roots are frequently below those required for inhibition of growth. The data here indicate that mixtures of phenolic acids in soil can inhibit growth of cucumber seedlings in an additive or antagonistic manner. We suspect that in nature it is a combination of phenolic acids that brings about an inhibitory effect and not individual phenolic acids. The fact that individual components in a mixture may be antagonistic in their action does not detract from the fact that mixtures have a greater impact on growth, unless, of course, the compounds are completely antagonistic (i.e., $\beta_5 = -1$). Finally, the data clearly indicate that soil characteristics such as acidity may influence the effects of phenolic acids and mixtures of phenolic acids on plant growth.

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SHORT-TERM INDUCTION OF ALKALOID PRODUCTION IN LUPINES

Differences Between N₂-Fixing and Nitrogen-Limited Plants

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Abstract—We used N₂-fixing and nonfixing lupines to examine the effects of plant nutrition on short-term alkaloid production in damaged leaves. Three different treatments were used: damaged leaves from N₂-fixing plants; undamaged leaves from these damaged, N₂-fixing plants; and damaged leaves on nitrogen-limited, nonfixing plants. Relative to controls, alkaloids increased in concentration more quickly in the N₂-fixing than in the nitrogen-limited plants. The magnitude of this increase in alkaloids was correlated with the initial alkaloid concentration. These results suggest that nitrogen-rich plants may benefit from faster and higher alkaloid induction than nitrogen-limited plants. In addition, the detailed dynamics of individual alkaloids are consistent with earlier proposals for the mechanism of lupine alkaloid induction.

Key Words—Plant defense, induced defense, leaf damage, quinolizidine alkaloids, nitrogen fixation, *Lupinus succulentus*.

INTRODUCTION

Just as animals increase their immunity when infected by disease, plants beset by herbivores often quickly become less palatable or less nutritious (Tallamy, 1985; Broadway et al., 1986). One reason for this decline in palatability is that many plants produce defenses, called *induced* defenses, following herbivore attack (Rhoades, 1983; Schultz, 1988). The defenses of both animals and plants are also profoundly affected by nutrition (Gershenson, 1984; Larsson et al., 1986). But in plants, this interaction between nutrients and defenses is only

partly understood. Previous studies of the interactions between environmental nutrients and defenses have all focused on *constitutive* defenses, those present prior to herbivore attack. In this report, we describe interactions between induced defenses and plant nutrition.

Plant nutrition influences so many aspects of plant growth and development that it would be surprising if induced defenses were not also affected (Chapin, 1980). Indeed, several studies have reported significant interactions between the effects of nutrients and defoliation on plant defenses (Bryant, 1987; Johnson et al., 1987), but these previous studies investigated chemical changes that developed weeks after defoliation, not those that occurred within the first few hours.

We have focused on a nutritional factor that could readily alter the short-term induction of plant defenses: nitrogen availability. As an example, consider how nitrogen availability might regulate alkaloid induction. In low-nitrogen soils, plants are likely to have low levels of most nitrogenous molecules including the enzymes of alkaloid biosynthesis and the amino acids that are the precursors of many plant alkaloids (Waller and Nowacki, 1978). Nitrogen-limited plants therefore should mount a lower and slower alkaloid response to damage than plants with an adequate nitrogen supply. Of course, other factors such as enzyme inhibitors could also regulate toxic chemical synthesis, but environmental nutrient levels may affect these factors as well.

To examine whether nutrient availability does regulate induced responses in plants, we studied alkaloid levels in plants with and without symbiotic N_2 fixation. For our model N_2 -fixing plant we chose the annual *Lupinus succulentus* Dougl. ex Koch. (Fabaceae). Lupines are an appropriate study plant because their major defenses against leaf-eating herbivores, the quinolizidine alkaloids, are very sensitive to N_2 fixation and because the quinolizidine alkaloids are inducible (Wink, 1983; Johnson et al., 1987). In addition, by studying *L. succulentus*, we can contrast our results for short-term alkaloid induction with a previous study of long-term induction in the same species (Johnson et al., 1987). This earlier study found that, for plants relying on N_2 fixation, a three-week regime of partial defoliation ultimately led to a decline in leaf alkaloid concentrations. Thus, N_2 -fixing lupines may have a biphasic alkaloid response to defoliation, with rapid initial induction followed by a long-term decline in alkaloid concentrations.

METHODS AND MATERIALS

Plants and Rhizobia. Two treatment groups of *Lupinus succulentus* plants were grown in a greenhouse in sand culture during February and March 1987. One group of plants was inoculated with *Bradyrhizobium* sp. N_2 -fixing bacteria

isolated from lupines (Nitragin Co., Milwaukee, Wisconsin) and fertilized with a nitrogen-free nutrient solution. These plants were quite vigorous and had none of the red pigmentation characteristic of nitrogen-stressed *L. succulentus*. A second group, the low-NO₃-plants, were fertilized with a similar nutrient solution supplemented with 0.2 mM KNO₃. The plants in this group were clearly stunted, and both their petioles and adaxial leaf surfaces were red. Other general characteristics of plants grown under these nutrient treatments and the nutrient solution compositions are given in Johnson et al. (1987).

Experimental Design. The low-NO₃-plants were allocated to two groups: plants with damaged leaves and undamaged controls. Leaves from two to three of these stunted, small-leaved plants had to be pooled for each alkaloid analysis, yielding eight samples for the controls and nine samples of damaged leaves. The N₂-fixing plants were also allocated to damaged ($N = 22$ plants) and control groups ($N = 17$), but because these plants were much larger than the low-NO₃-plants, a third treatment was added. This last treatment involved harvesting undamaged leaves from previously damaged plants ($N = 22$).

To control for the large within-plant and among-plant variability in *L. succulentus* alkaloids, we tracked the alkaloid dynamics of the individual palmately compound leaves. Several fully expanded leaves were chosen from each plant, and these leaves were sampled at 1200 hr and then 4 and 24 hr later. At each sampling, two to three leaflets were removed from each leaf by snipping the narrow leaflet base, a procedure that causes no detectable alkaloid induction (personal observation). The harvested leaves were immediately placed on dry ice and stored frozen at -15°C until analysis. For the N₂-fixing plants, leaflets were pooled and analyzed separately for each plant and harvest time; for the smaller low-NO₃-plants, leaflets were analyzed as pools of several plants, as described above.

Leaves were damaged by pressing a screw-gripper on all of the leaflets of each designated leaf, generating a grid of wounds over the entire leaf spaced at 36/cm². This method was chosen because it left all of the leaflet behind for subsequent alkaloid analysis. The alkaloid induction caused by this method is similar to the induction caused by the more conventional method of leaflet-clipping (unpublished results).

Chemical Analyses. Alkaloids were extracted from weighed, dried leaf material into 0.5 M HCl, with cinchonidine HCl added as an internal standard to 0.1% dry leaf weight. The resulting extract was made alkaline with conc. NH₄OH, separated with CH₂Cl₂, and then concentrated under nitrogen. These concentrates were injected into a Shimadzu Mini-2 gas chromatograph equipped with a flame ionization detector and DB-1 megabore capillary column (J&W Scientific). Alkaloids were eluted by temperature programming from 150 to 300°C followed with 13 min at 300°C, and GC-mass spectrometry and co-GC with known samples were used for identification (Johnson et al., 1987).

Statistical Analysis. Most of the statistical analyses were done using variates that represented the changes in alkaloids over time. To examine the relative induction of alkaloids, the levels of each alkaloid after damage were expressed as a fraction of their initial concentrations, i.e., the ratio between final and initial alkaloids. These ratios were then log-transformed, and individual plants were represented as vectors of these ratios for the alkaloids tetrahydro-rhombifoline, lupanine, and the pooled *cis*- and *trans*-cinnamic acid esters of 13-hydroxylupanine. These particular alkaloids were chosen because they were present in all plants and accounted for 75–100% of the total plant alkaloids.

The overall effects of damage were examined for the three treatments (N_2 -fixing, low NO_3^- , and undamaged N_2 -fixing) with multivariate analysis of variance (MANOVA) of damage treatment vs. controls. To test for differences in the alkaloid dynamics of plants with different nitrogen sources, the changes in alkaloids over each subinterval (0–4 hr and 4–24 hr) were compared by two-way MANOVA of damage (damage/control) and nitrogen source (low NO_3^- and N_2 -fixing). Since the changes in alkaloids in both subintervals are calculated using the alkaloid concentrations at 4 hr, the variates for the two subintervals are not independent of each other. We therefore adjusted the significance levels of these MANOVAs for two nonindependent comparisons using the Dunn-Sidak method (Ury, 1976). The dynamics of individual alkaloids were examined using the same variates as in the MANOVAs, i.e., variates expressing the change in alkaloids over each time interval. Instead of analyzing these variates by MANOVAs, however, two-way ANOVAs were used to test the effects of damage and alkaloid type for each different treatment (low NO_3^- , N_2 -fixing, and undamaged N_2 -fixing). We also tested for alkaloid differences among treatments at the beginning of the experiment using one-way (damage) or two-way (damage \times nitrogen source) MANOVAs of the absolute alkaloid concentrations.

All statistical operations used either the GLM (general linear models) or the CORR procedure (product-moment correlations) of SAS (SAS Institute, Cary, North Carolina). Significance values for MANOVAs are derived from the *F* approximation of Pillai's trace.

RESULTS

Induced Responses in N_2 -fixing and low- NO_3^- Plants Relative to Controls.

Changes over All Alkaloids. Leaf damage caused a 55% increase in alkaloids relative to controls for both the N_2 -fixing and low NO_3^- plants, and a 33% increase in undamaged leaves on damaged N_2 -fixing plants (Figure 1). This increase in alkaloids was highly significant for the N_2 -fixing treatment (MAN-

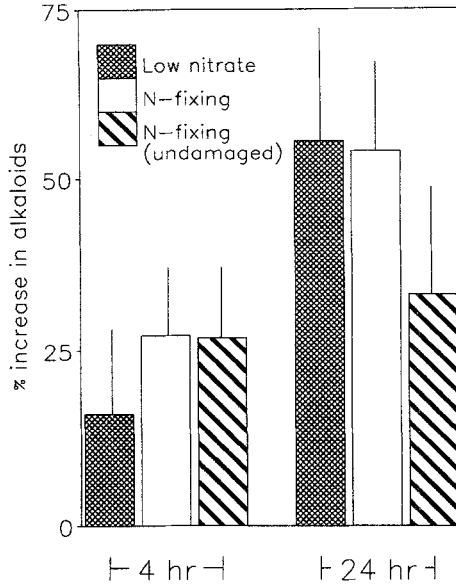


FIG. 1. Alkaloid induction in *Lupinus succulentus*. Bars represent the treatment means of the values of $(A_i/C_i - A_0/C_0) \times 100$ for each plant, where A and C are the total alkaloid concentrations of experimental and control plants, respectively, and the subscript indicates the time since leaves were damaged. Error lines represent one standard error.

OVA, $P = 0.009$), marginally significant for low- NO_3^- plants ($P = 0.058$), and nonsignificant for the undamaged leaves on damaged, N_2 -fixing plants ($P = 0.66$).

Overall, the 24-hr alkaloid induction in damaged leaves did not differ significantly between the low- NO_3^- and N_2 -fixing treatments, as shown by the nitrogen source \times damage interaction in a two-way MANOVA of 24-hr changes in alkaloids (Table 1). The significant alkaloid induction noted above is also shown in this two-way MANOVA, and a further significant effect is revealed, that of nitrogen source (Table 1). Nitrogen source was a significant factor because the low- NO_3^- plants, both damaged and control, tended to increase in alkaloid levels relative to the N_2 -fixing plants (discussed below).

Although the N_2 -fixing and low- NO_3^- plants had similar induced responses overall, the timing of their responses differed. More than half the alkaloid increase in the N_2 -fixing plants occurred within 4 hr, whereas most of the response in the low- NO_3^- plants occurred later, between 4 and 24 hr (Figure 1). These treatment differences in the timing of induction were statistically significant in the period between 4 and 24 hr, as shown by a two-way MANOVA of

TABLE 1. TWENTY-FOUR-HOUR CHANGES IN ALKALOIDS ANALYZED BY TWO-WAY MANOVA OF LEAF DAMAGE AND NITROGEN SOURCE^a

Effect	df	F	P
Damage	3,48	5.25	0.007
N source	3,48	11.34	<0.0002
N source × damage	3,48	0.84	0.73

^aF statistics are from Pillai's trace.

nitrogen source and damage treatment (nitrogen source × damage interaction, $P = 0.045$).

A second way of analyzing induction is to determine if induction is proportional to initial alkaloid content. We did this by examining the correlations between the initial alkaloid levels and the arithmetic change in alkaloids following damage, with all data divided by the appropriate controls. That is, we determined the correlations between $(E_{24}/C_{24} - E_0/C_0)$ and E_0/C_0 , where E_i and C_i are the concentrations of alkaloids after i hours in experimental and control plants, respectively.

For both of the N_2 -fixing treatments, induction was correlated with initial alkaloid content ($r = 0.69$, $P = 0.0003$, damaged leaves; $r = 0.41$, $P = 0.057$, undamaged leaves on damaged plants). This indicates that the leaves with the highest alkaloid levels also had the highest increases in alkaloids relative to controls. But no significant correlation was found for the low- NO_3^- plants ($r = 0.12$, $P = 0.8$), perhaps because the low- NO_3^- plants spanned a smaller range of initial alkaloid concentrations. In the N_2 -fixing plants, alkaloid levels were distributed fairly uniformly across two orders of magnitude; but in the low- NO_3^- plants, the highest levels were only three times the lowest.

Induction of Individual Alkaloids. The dynamics of individual alkaloids tended to parallel those of total alkaloids with the exception of 17-oxosparteine and the hydroxylupanine esters (Figure 2). These two alkaloids are, respectively, the first and perhaps the last alkaloids in the quinolizidine biosynthetic pathway in *L. succulentus* (Wink et al., 1981). The variation in the levels of individual alkaloids is quite high, however, so that significant differences among alkaloids are only found in the N_2 -fixing plants at 0–4 hr (Figure 2A, $P = 0.01$ for alkaloid type × damage interaction in a two-way ANOVA of alkaloid type and damage).

Dynamics of Alkaloid Production.

At the beginning of the experiment, alkaloids differed significantly between the N_2 -fixing and low- NO_3^- groups ($P < 0.0001$, MANOVA), but not among the

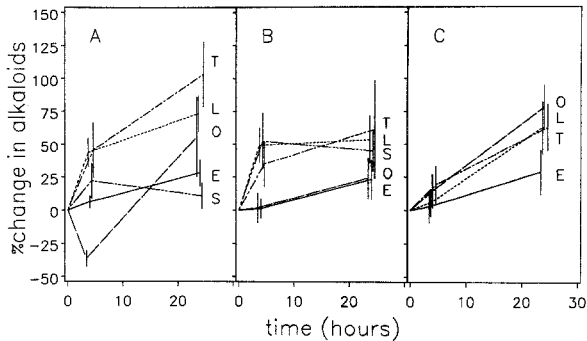


FIG. 2. The dynamics of individual alkaloids following leaf damage treatments: (A) damaged N₂-fixing; (B) undamaged N₂-fixing; (C) low NO₃⁻. The means for each treatment and time were calculated using the formula from Figure 1 applied to individual alkaloids. Errors are one standard error. S = sparteine, T = tetrahydrohombifoline, O = 17-oxosparteine, L = lupanine, and E = *cis*- and *trans*-cinnamic acid esters of 13-hydroxylupanine.

damage treatments within each group (N₂-fixing, *P* = 0.3; low-NO₃⁻, *P* = 0.7; both MANOVA; Table 2). This significant effect of nitrogen source was due to differences in alkaloid composition rather than to differences in absolute alkaloid concentrations. The N₂-fixing plants had a relative alkaloid composition of 32:26:17:24 tetrahydrohombifoline–lupanine–sparteine–lupanine esters, whereas low-NO₃⁻ plants were 72:3:1:24 (both normalized to add to 100). These relative alkaloid compositions are those expected for N₂-fixing and low-NO₃⁻ plants, respectively (Johnson et al., 1987).

TABLE 2. MEANS AND STANDARD DEVIATIONS OF LUPINE ALKALOIDS FROM DIFFERENT NUTRIENT AND DAMAGE TREATMENTS

Treatment	Harvest time (hr)		
	0	4	24
N ₂ -fixing			
Damaged	0.125 ^a (0.158)	0.117 (0.159)	0.118 (0.157)
Control	0.083 (0.081)	0.059 (0.048)	0.056 (0.045)
Undamaged, from damaged plants	0.081 (0.096)	0.070 (0.081)	0.068 (0.081)
Low NO ₃ ⁻			
Damaged	0.068 (0.025)	0.072 (0.021)	0.101 (0.041)
Control	0.084 (0.046)	0.086 (0.058)	0.088 (0.041)

^a Alkaloids are percent of leaf dry weight.

The changes in the actual leaf alkaloid concentrations following damage contrast somewhat with the results described in the previous section. We previously showed that alkaloids were induced relative to undamaged controls, but, for the N_2 -fixing plants, the absolute effect of damage was to halt the decline in alkaloids seen in the control plants (Table 2). In contrast, the low- NO_3^- plants showed similar alkaloid dynamics both relative to controls and for absolute alkaloid concentrations.

DISCUSSION

Nitrogen Availability, Induced Responses, and Plant-Herbivore Interactions. Nitrogen availability affects virtually all aspects of plant physiology and chemistry, and it would be surprising if induced responses were not affected as well. Indeed, our results suggest that N_2 -fixation, by providing plentiful nitrogen, can regulate both the pace and magnitude of induced chemical changes in lupines. This interpretation does not readily explain all of our experimental observations, however. In particular, it does not explain why the alkaloids of N_2 -fixing plants tended to decline after the first harvest (Table 2). Other reasons that low- NO_3^- and N_2 -fixing lupines may have different alkaloid dynamics include differences in leaf age, plant developmental stage, and leaf and plant photosynthetic rates (Wink and Hartmann, 1982; Wink and Witte, 1985; Johnson and Bentley, 1989). These other mechanisms are testable alternatives to our hypothesis that the differences between low- NO_3^- and N_2 -fixing plants were directly due to differences in nitrogen availability.

In theory, the effects of N_2 -fixation on alkaloid induction should have straightforward consequences for lupine plant-herbivore interactions. N_2 -fixing plants will have rapid alkaloid induction in damaged leaves and perhaps in undamaged tissue as well, although the changes in undamaged leaves were not significant in this study. The quinolizidine alkaloids are potent toxins and feeding deterrents, serving as the principal lupine defenses against leaf-eating herbivores (e.g., Wink, 1984). Therefore, the faster the induction, the more effective the deterrence to further feeding by herbivores; however, as Fowler and Lawton (1985) have cautioned, the simple existence of induction does not prove its ecological and evolutionary importance.

The positive correlation between initial leaf alkaloids and the magnitude of alkaloid induction suggests a second consequence of N_2 fixation. Plants with abundant available nitrogen, either from N_2 fixation or from the soil, generally have higher alkaloids as well as higher protein than nitrogen-starved plants (Johnson et al., 1987). Young leaves also have high protein and alkaloid levels. Hence, the absolute induction of alkaloid production should be greater in pro-

tein-rich plants and foliage. This may be particularly important for lupines, since a given concentration of lupine alkaloids is less toxic at higher protein concentrations (for *Spodoptera eridania*, Lepidoptera; Johnson and Bentley, 1988).

The short-term alkaloid induction in N_2 -fixing plants stands in marked contrast to the long-term effects of damage. In a previous study, we showed that defoliation eventually depressed alkaloid levels in *L. succulentus* plants relying on N_2 -fixation (Johnson et al., 1987). Thus, the short-term burst of alkaloid production reported here cannot be maintained in the face of repeated defoliation.

Regulation of Alkaloid Induction by Nitrogen Availability. Because of the manifold effects of nitrogen availability on plant growth and physiology, many different mechanisms could account for the effects of nitrogen availability on induced responses. For example, nitrogen availability may affect average leaf age, plant developmental stage, or, as we suggested in the Introduction, the levels of alkaloid precursors or biosynthetic enzymes.

Of these latter two mechanisms, it is more likely that induction is regulated by the biosynthetic machinery than by the size of precursor pools. For example, if new enzymes must be synthesized before induction can occur, then protein translation inhibitors should halt induction. A study by Wink (1983) showed exactly this for leaves of *Lupinus polyphyllus*. The alternative, regulation by precursor availability, is disputed by circumstantial evidence from the data presented here. If induction were limited because precursors became depleted, then nitrogen-starved plants should have especially low levels of 17-oxosparteine, the precursor for all other *L. succulentus* alkaloids (Wink et al., 1981). In fact, our experiments gave exactly the opposite result. The low- NO_3^- lupines, with the slowest induction, had the highest concentrations of 17-oxosparteine, whereas the N_2 -fixing plants had the fastest induction and the lowest 17-oxosparteine levels (Figure 2). Thus, precursor depletion more likely regulated the induction of the N_2 -fixing than the low- NO_3^- plants.

SUMMARY

We showed that plant nitrogen source could affect both the timing and the magnitude of induced alkaloid production, perhaps because nitrogen stress reduced the levels of the enzymes of alkaloid biosynthesis.

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IDENTIFICATION OF SEX PHEROMONE COMPONENT OF SPRUCE BUDMOTH *Zeiraphera canadensis*¹

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Abstract—The analyses of virgin female sex pheromone gland extracts by gas chromatography (GC), GC-electroantennographic detection (GC-EAD) and GC-mass spectrometry (GC-MS) followed by field-trapping experiments, have identified (*E*)-9-tetradecenyl acetate (*E*9-14:Ac) as the primary sex pheromone component of the spruce budmoth, *Zeiraphera canadensis*. Dosages of 1.0–100.0 µg of *E*9-14:Ac impregnated in rubber septa provide effective trap baits.

Key Words—*Zeiraphera canadensis*, spruce budmoth, (*E*)-9-tetradecenyl acetate, sex pheromone, Lepidoptera, Tortricidae, Eucosminae, trapping.

INTRODUCTION

Two species in the budmoth group, the spruce budmoth, *Zeiraphera canadensis* Mutuura and Freeman, and the purplestriped shootworm, *Zeiraphera unfortunana* Powell [= *Z. destitutana* (Walker)], are sympatric in New Brunswick and feed on white spruce [*Picea glauca* (Moench) Voss]. These species feed on

¹Lepidoptera: Tortricidae: Eucosminae.

developing apical shoots (Martineau, 1984), which results in reduced tree height and bole deformation.

The primary sex pheromone component of *Z. unfortunana* has recently been identified as (*E*)-9-dodecenyl acetate (*E*9-12:Ac) (Silk et al., 1988). The pheromone for *Z. canadensis* has not been identified; however, (*E*)-9-tetradecenyl acetate (*E*9-14:Ac) has been found to be electrophysiologically active and effective in trapping experiments (Turgeon and Grant, 1988).

We report here the identification of *E*9-14:Ac as the primary sex pheromone component of *Z. canadensis*. This was achieved by analyses of sex pheromone gland extracts using GC, GC-EAD, and GC-MS and field-trapping experiments.

METHODS AND MATERIALS

Insects. During 1986, white spruce foliage (50-cm branches), infested with *Z. canadensis* and *Z. unfortunana* larvae, was collected. The branches were suspended over trays of moist vermiculite in rooms at constant temperature (25°C) and relative humidity (70–80%). Mature larvae subsequently dropped into the trays where pupation occurred.

The two species were then initially separated on the basis of pupal size (*Z. unfortunana* is larger than *Z. canadensis*), sexed (Turgeon, 1985), and emerged adults were separated by differences in thoracic and wing maculations. Adults were maintained in small cages under the same environmental conditions as were pupae (Silk et al., 1988).

Collection of Pheromone. Virgin female *Z. canadensis* and *Z. unfortunana* were held one to two days postemergence, and their sex pheromone glands were manually everted and excised during the first 3–4 hr of scotophase. Four hundred twenty *Z. canadensis* glands were placed in Spectrograde hexane, and 40 glands were placed in chloroform–methanol (2:1 v/v) for subsequent methanolysis (Bjostad and Roelofs, 1984). In addition, 21 *Z. unfortunana* glands were also placed in chloroform–methanol for methanolysis. *Z. canadensis* glands, in hexane, were sonicated for 30 min, filtered through clean glass wool, and the extract was then used for subsequent GC, GC-EAD, and GC-MS analyses. All gland extracts were stored in solvent at –10°C prior to analyses.

GC-EAD Analysis. Portions of the pheromone gland extract in hexane were analyzed on a GC-EAD system (Struble and Arn, 1984; Silk et al., 1988) using detector antennae of 2- to 3-day-old male *Z. canadensis* and *Z. unfortunana* moths; EAD responses and GC retention times were also recorded from synthetic standards.

Fatty Acid Analyses. Forty virgin female glands of *Z. canadensis* in chloroform–methanol (2:1) were sonicated for 20 min, extracted as above, and the

extracts were subjected to alkaline methanolysis (Bjostad and Roelofs, 1984) followed by reaction with dimethyl disulfide (DMDS) (Dunkelblum et al., 1985). These DMDS adducts, in hexane, were analyzed by GC-MS as detailed below. Methanolized gland extracts of *Z. unfortunana* were derivatized and analyzed similarly.

GC and GC-MS Analysis. GC analyses were performed as previously described (Silk et al., 1988). The following columns were used: Column A, 30 m \times 0.32 mm DB-5 (Chromatographic Specialties Ltd.); Column B, 30 m \times 0.32 mm SPB-5 (Supelco); Column C, 50 m \times 0.30 mm Superox FA (Mandel Scientific Ltd.).

GC-MS analyses on extracts, adducts, and synthetics were performed as previously described (Silk et al., 1988) using column A.

Chemicals. Chemicals used in the above procedure were obtained from Chem. Samp. Co., Columbus, Ohio; Koken Fine and Aromatic Chemicals, Tokyo, Japan; or were synthesized in our laboratory using standard techniques; all chemicals were 98% pure (<0.1% opposite isomers) as determined by capillary GC and used without further purification.

Field Testing. Field-trapping studies were carried out in 1986 in the same plantations where larvae were collected. Plots ca. 50 m apart were chosen in homogeneous white spruce stands that were planted in 1976.

Two trapping experiments were conducted; the first was conducted for 11 nights (July 19–30) in a 6 (treatments) \times 10 (replicates) randomized block. The second experiment was conducted for 10 nights (July 20–30) in a 5 (treatments) \times 5 (replicates) randomized block. In both trapping experiments, Pherocon-1C traps were baited with red rubber septa lures (Arthur H. Thomas Co.) and suspended from white spruce foliage ca. 1.5 m from the ground (mid-crown) with 25-m spacings between traps.

All moths caught in the traps were identified (*Z. canadensis* and *Z. unfortunana* males) by examining their genitalia (Mutuura and Freeman, 1966).

Log-transformed data were analyzed by two-way ANOVA and means compared using Tukey's HSD multiple-comparison test.

RESULTS

Chemical and GC-EAD Analyses. From the 420 *Z. canadensis* glands obtained for analysis, aliquots containing ca. 50 female-gland equivalents in hexane (2 μ l) were injected on columns A, B, and C for GC-EAD analysis using male *Z. canadensis* as detector antennae. Several gland components produced small EAD responses, but only one discernible GC peak produced a large EAD response (on all columns). The GC retention time (columns A and B) of this EAD-active component closely matched that of synthetic 9-tetradecenyl

acetate (9-14:Ac). The retention time of the component suggested the *E* configuration of the double bond when GC-EAD analysis was carried out on column C (*E* and *Z* isomers well resolved).

GC-EAD responses of the gland component (largest EAD response) and synthetic compounds are shown in Table 1. For comparison purposes, GC-EAD responses of male *Z. unfortunana* antennae to several synthetics are also presented (Silk et al., 1988) (Table 1). The 9-14:Ac clearly gave medium to large EAD responses, while the saturated analogs, at 1-ng dosages, elicited no response in *Z. canadensis* antennae but a range of responses in *Z. unfortunana* antennae (Silk et al., 1988) (Table 1).

GC-MS analysis was carried out on the same DB-5 capillary column with the remaining *Z. canadensis* gland extract (ca. 120 gland equivalents). One weakly EAD-active region corresponded in retention time and MS characteristics to *E*9-12:Ac [*m/e* 61 ($\text{CH}_3\text{COOH}_2^+$) 68, 82, 166 ($\text{M}^+ - 60$)]. The MS scan corresponding to the region of highest EAD response corresponded to *E*9-14:Ac

TABLE 1. MEAN EAD RESPONSE OF MALE *Z. canadensis* AND *Z. unfortunana* ANTENNAE TO SOME SYNTHETICS AND PHEROMONE GLAND EXTRACTS

Stimulus ^a	EAD Response (mV)	
	<i>Z. canadensis</i>	<i>Z. unfortunana</i>
<i>E</i> 11-14:Ac	0.4 ± 0.5 (<i>N</i> = 3) ^b	0 ^c
<i>E</i> 9-14:Ac	3.0 ± 1.6 (<i>N</i> = 10)	1.6 ± 1.3 (<i>N</i> = 6)
<i>E</i> 7-14:Ac	0.3 ± 0.3 (<i>N</i> = 3)	0
<i>E</i> 7-12:Ac	0.2 ± 0.4 (<i>N</i> = 3)	0
<i>E</i> 9-12:Ac	2.5 ± 1.3 (<i>N</i> = 5)	4.2 ± 1.5 (<i>N</i> = 5)
<i>E</i> 9-14:OH	1.8 ± 1.1 (<i>N</i> = 2)	0
<i>Z</i> 11-14:Ac	0.5 ± 0.09 (<i>N</i> = 2)	0
<i>Z</i> 9-14:Ac	2.2 ± 0.18 (<i>N</i> = 2)	0
<i>Z</i> 7-14:Ac	0.4	0
<i>Z</i> 7-12:Ac	0.7	0
<i>Z</i> 9-12:Ac	2.1 ± 1.7 (<i>N</i> = 3)	2.8 ± 0.7 (<i>N</i> = 3)
12:Ac	0	2.1 ± 0.9 (<i>N</i> = 3)
14:Ac	0	0.6
12:OH	0	0.4
14:OH	0	0
Major gland component ^d	2.7 (46 FE)	2.3 (5 FE)

^a 1 ng stimulus, i.e., 2 ng injected on GC.

^b $\bar{X} \pm 1$ SD.

^c Not distinguishable from background response (ca. 0.05 mV).

^d Forty-six female equivalents (FE); on SPB-5 capillary column, 50-210°C at 8°/min.

[*m/e* 61 (CH₃COOH₂⁺) 67, 82 (base) 96, 110, 124, and 194 (M⁺-60)]. Insufficient material was available for double-bond confirmation by microchemical means.

Additional supportive evidence for double-bond assignment resulted from the fatty acid analyses that are shown in Table 2 (note the presence of 11-14:Me in *Z. unfortunana*). As expected, 9-14:Me (methyl 9-tetradecenoate) was detected, and this fatty acid is the presumed precursor to the 9-14:Ac (Roelofs and Bjostad, 1984), the primary EAD-active gland component. In addition, 9-12:Me (methyl 9-dodecenoate) was detected as expected from EAD and MS data. These data support the double-bond assignment in the gland-derived 9-dodecenyl and 9-tetradecenyl acetates.

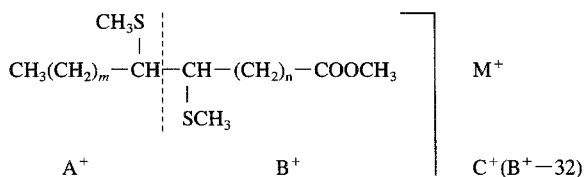
GC-EAD analysis (column B) of *Z. canadensis* sex pheromone gland

TABLE 2. FATTY ACID ANALYSES OF *Z. canadensis* AND *Z. unfortunana* VIRGIN FEMALE SEX PHEROMONE GLAND EXTRACTS

Fatty acid ^a	MS ^b characteristics (<i>m/e</i>)				Relative amount per gland ^c
	M ⁺	A ⁺	B ⁺	C ⁺	
<i>Z. canadensis</i>					
Δ9-12:Me	306	89	217	185	VS
Δ6-14:Me	334	159	175	143	VS
Δ9-14:Me	334	117	217	185	VS
Δ9-16:Me	362	145	217	185	L
Δ9-17:Me	376	159	217	185	VS
Δ9-18:Me	390	173	217	185	VL
<i>Z. unfortunana</i>					
Δ9-12:Me		89	217	185	VS
Δ6-14:Me	334	159	175	143	VS
Δ9-14:Me	334	117	217	185	VS
Δ11-14:Me	334	89	245	213	VS
Δ9-16:Me	362	145	217	185	L
Δ9-17:Me					
Δ9-18:Me	390	173	217	185	VL

^aGenerated as methyl esters in the methanolysis step; Δ9-12:Me methyl 9-dodecenoate.

^bSee Dunkelblum et al. (1985)



^cVL, very large (ca. 1 μg); L, large (ca. 100 ng); VS, very small (<0.1 ng).

extract (4.6 female equivalents) was also carried out using male *Z. unfortunana* antennae (*Z. unfortunana* strongly responds to *E9-12:Ac* and moderately to *E9-14:Ac*; Silk et al., 1988). EAD responses were recorded to two GC peaks coincident with the retention times of synthetic *E9-12:Ac* and *E9-14:Ac* under identical GC conditions indicating that these two compounds were indeed present in *Z. canadensis* sex pheromone gland extracts.

In summary, the evidence from GC, GC-EAD, and GC-MS analyses supports the assignment of *E9-14:Ac* as the major EAD-active component in *Z. canadensis* sex pheromone glands. In addition, MS and EAD data support the assignment of a smaller amount (<5% of *E9-14:Ac*) of *E9-12:Ac* in sex pheromone gland extracts. Other weakly EAD-active components were not identified.

Field Testing. The results of the first dosage and blend test (1986) indicated that all treatments were significantly different from controls (Table 3) ($P < 0.05$) but were not significantly different from each other. Clearly, in this test, *E9-14:Ac* effectively trapped *Z. canadensis* males, although a dose-response effect was not observed in these trapping experiments. Also, the addition of 10% *Z9-14:Ac* or 10% *E9-14:OH* to 1 μg of *E9-14:Ac* did not significantly affect trap captures ($P > 0.05$).

Our study with pure components and blends of *E9-14:Ac* and *E9-12:Ac* indicate that both *E9-14:Ac* and *E9-12:Ac* at the 10- μg dosage, were effective in capturing *Z. canadensis* and *Z. unfortunana*, respectively (Table 4). In the three blends of the two acetates tested, higher concentrations of a specific chemical constituent resulted in higher trap captures of the species specific to the given acetate (e.g., the higher the proportion of *E9-12:Ac*, the higher the proportion of *Z. unfortunana* captured). Addition of *E9-12:Ac*, identified as a

TABLE 3. TRAP CAPTURE OF MALE *Zeiraphera canadensis* IN PHEROCON-1C TRAPS BAITED WITH DIFFERENT DOSAGES OF *E9-14:Ac* AND BLENDS OF *E9-14:Ac* WITH *Z9-14:Ac* AND *E9-14:OH*

Compounds	Dosage (μg)	Trap capture (mean \pm 1 SD) ^a
<i>E9-14:Ac</i> (1)	1	37 \pm 13.0a
<i>E9-14:Ac</i>	10	29 \pm 21.4a
<i>E9-14:Ac</i>	100	38 \pm 15.9a
(1) + 10% <i>Z9-14:Ac</i>	1	47 \pm 24.9a
(1) + 10% <i>E9-14:OH</i>	1	36 \pm 39.2a
Check (blank trap)		11 \pm 7.8b

^aMeans followed by same letter are not significantly different; $P > 0.05$.

TABLE 4. MEAN NUMBER OF *Zeiraphera canadensis* AND *Z. unfortunana* CAUGHT IN TRAPS BAITED WITH PROPORTIONAL BLENDS OF E9-14: Ac AND E9-12: Ac^a

Blend (%)		<i>Z. canadensis</i> ($\bar{X} \pm 1$ SD)	<i>Z. unfortunana</i> ($\bar{X} \pm 1$ SD)
E9-14: Ac	E9-12: Ac		
100	0	15 \pm 10.3	0 \pm 0
80	20	9 \pm 6.9	3 \pm 2.3
50	50	5 \pm 3.3	16 \pm 7.0
20	80	4 \pm 3.1	38 \pm 7.1
0	100	3 \pm 1.9	52 \pm 27.8

^a A total of 10 μ g (see text for details).

gland component, to E9-14: Ac, therefore, did not increase male *Z. canadensis* trap capture at the tested dosages.

DISCUSSION

Field-trapping data in this work indicate that E9-14: Ac, impregnated in rubber septa, in the 1- to 100- μ g range, effectively traps *Z. canadensis* males. A dose-response, however, could not be demonstrated, and this could be explained by mid-crown placement of traps. Turgeon and Grant (1988), for example, clearly showed a dose-response with E9-14: Ac when traps were placed at the tops of trees.

Addition of either Z9-14: Ac or E9-14: OH (Table 3) to E9-14: Ac does not affect trap capture. Traps with blends of E9-12: Ac (a sex pheromone component of *Z. unfortunana*; Silk et al., 1988) and E9-14: Ac capture both species (Table 4), but the pure components appear to be reasonably specific in capturing one species only. The role of E9-12: Ac in the sex pheromone glands of *Z. canadensis* is, therefore, unknown at this time.

Several lepidopteran sex pheromone components have been shown to be biosynthesized by reaction of fatty acids with two key enzyme systems that are found only in sex pheromone glands (Roelofs and Bjostad, 1984). These are a microsomal β -oxidation system, which yields limited chain shortening by two carbons, and a Δ 11-desaturase system. The characterized pheromones in the coniferophagous *Choristoneura* budworms, for example, can all be generated by these two enzyme systems starting from hexadecanoic acid (Wolf and Roelofs, 1987; Silk and Kuenen, 1988). The comparison of monounsaturated fatty acids in these two *Zeiraphera* species is interesting in this regard (Table 2): methyl 9-dodecenoate and methyl 9-tetradecenoate were identified in both spe-

cies, and corresponding acids are the presumed biosynthetic precursors to the identified unsaturated acetate pheromones. Other identified fatty acids may represent precursors to minor pheromone components. A plausible biosynthetic route to these acetate pheromones in both species is shown in Fig. 1. Both would be generated from hexadecanoate. For *Z. canadensis*, the $\Delta 11$ -desaturase (*E*-specific) inserts the double bond followed by limited β -oxidation to a (*E*)-9-tetradecenoyl moiety. Reduction and acetylation would produce the required *E*9-14:Ac. With *Z. unfortunana*, β -oxidation would occur first followed by $\Delta 11$ -desaturation (*E*-specific); a second β -oxidation step, then reduction and acetylation would yield *E*9-12:Ac. The fatty acid analyses reported in Table 2 support this scheme. Second, recent trapping studies in white spruce stands in Nova Scotia have found *E*11-14:Ac to be potentially active in capturing *Zeiraphera fortunana* (Butterworth and Silk, unpublished data). *Z. fortunana* is a relatively rare species found on white spruce in New Brunswick and Nova Scotia (Martineau, 1984). The male genitalis of *Z. fortunana* are morphologically

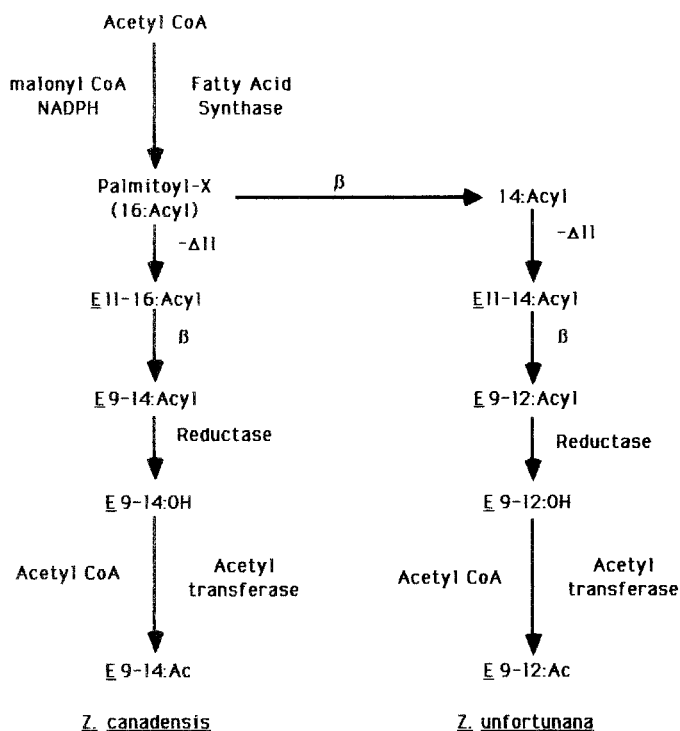


FIG. 1. Postulated divergent biosynthetic routes to the sex pheromones of *Z. canadensis* and *Z. unfortunana*. After Roelofs and Brown (1982) and Roelofs and Bjostad (1984). $-\Delta 11$ = $\Delta 11$ -desaturase; β = limited β -oxidation by two carbons.

very difficult to distinguish from *Z. unfortunana* and the two species are considered to be closely related (P. Dang, Biosystematics Institute, Agriculture Canada, personal communication). The fact that *E11-14:Ac* captures *Z. fortunana* is consistent with the postulated biosynthetic scheme outlined above and supports a close taxonomic relationship between *Z. unfortunana* and *Z. fortunana*.

Roelofs and Brown (1982) suggest a similar relationship between the two forms of *Z. diniana*, which occur on different host species in Europe. *Z. diniana* (larch form) produces *E11-14:Ac* (Roelofs et al., 1971; Guerin et al., 1984) while *Z. diniana* (cembran pine form) produces *E9-12:Ac* (Baltensweiler et al., 1978, Guerin et al., 1984).

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REPELLENT OF ROOT-KNOT NEMATODES FROM EXUDATE OF HOST ROOTS

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Abstract—A chemotaxis assay was developed to measure attraction or repulsion of infective juveniles of the root-knot nematode *Meloidogyne incognita* to exudates of host roots. The assay was sufficiently sensitive to measure the repellent activity of a 25- μ l sample of 7 mM NaCl. In tests of root exudate collected in a variety of ways from a variety of plants, avoidance responses were usually found but attraction was not. Extraction of the exudate from tomato with various organic solvents revealed that the repellent activity was highly polar. On Sephadex G-15 chromatography this exudate separated into two clearly defined peaks with apparent molecular weights of about 500 and 1000 daltons. The faster running peak (larger apparent size) contained much more activity than the other peak. Subsequent analysis of material from the larger peak via HPLC on a C18 column revealed a single peak of repellent activity. None of the chemical fractionations uncovered reproducible attractant activity.

Key Words—Root-knot nematode, *Meloidogyne incognita*, root exudate, bioassay, chromatography, repellent, chemotaxis, tomato.

INTRODUCTION

Nematodes are economically important pests in agriculture, but relatively little information is available on the mechanisms by which they locate host plants. In fact, there is little information on the cues used by any soil organism. The common assumption is that chemotaxis to chemicals released by the roots plays an important role (Steiner, 1925; Prot, 1980; Dusenbery, 1987b). One of the most widespread nematode pests is the root-knot nematode *Meloidogyne incognita*. As part of a series of investigations on the sensory behavior of infective

juveniles of this species (Diez and Dusenbery, 1989; Dusenbery, 1987a, 1988a-c; Goode and Dusenbery, 1985; Pline and Dusenbery, 1987; Pline et al., 1988), we have undertaken an investigation of its responses to root exudates from host plants. In addition, chemical characterization of the activity was initiated. A preliminary report on this work has been published previously (Diez and Dusenbery, 1986). A study of soybean cyst nematode responses to exudates of host roots has been reported (Papademetriou and Bone, 1982).

METHODS AND MATERIALS

Nematodes. The nematodes were grown on tomato plants (Rutgers cultivar) as previously described (Goode and Dusenbery, 1985). Eggs were collected from roots of infected plants using NaOCl (Hussey and Barker, 1973). Well-rinsed eggs were held on a nylon screen in a covered Petri dish with solution sufficient to just cover them. The solution consisted of sterile distilled water with 0.1% kanamycin sulfate and 0.05% gentamicin sulfate to control the growth of bacteria. Experiments indicated these antibiotics did not weaken the nematodes' responses to chemical stimuli as did hibitane. Each day the eggs were transferred to a fresh solution in another dish. Infective juveniles that had crawled through the screen were collected, rinsed, and concentrated in distilled water for use in the assay. The nematodes were generally used within two or three days of hatching when they are most infective (VanGundy et al., 1967).

Assay. Chemotaxis assays were conducted in small polystyrene boxes (2.0 × 2.0 × 6.0 cm) containing 4.0 ml of agar made up in deionized distilled water. Initially, 2% Bacto agar (Difco) was used (NaCl experiments); later 1.125% Bacto agar plus 0.375% Nobel agar was used to improve the way sample drops spread on the surface. After the agar was poured, the boxes were covered with paper towels to reduce air currents and the boxes were arranged to minimize temperature gradients while the agar cooled. This procedure promoted even hardening of the agar. After several hours, a sample (25 μ l with pure Bacto agar, 60 μ l with mixed agars) was spotted on the agar near one end and an equal volume of water or appropriate control solution was spotted on the other end. The boxes were again covered and allowed to sit for several more hours to allow the sample to diffuse into the agar. (This period generally varied from 5 to 10 hr between sets of experiments, but varied less than an hour within a given set of experiments. It had only a small effect on sensitivity as seen in Figures 1 and 2.) Several hundred nematodes were then applied to the agar surface in a minimal volume (10–20 μ l) of water. The plastic boxes were then placed side by side in a single row on a metal (copper or aluminum) plate (0.47 × 7.5 × 46 cm) that rested inside an insulated box, with internal dimensions

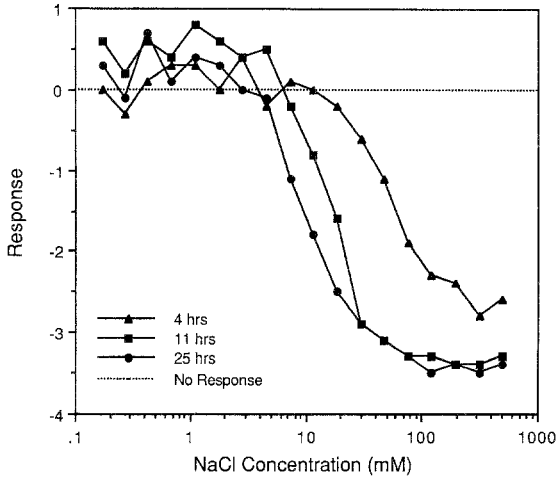


FIG. 1. Response to NaCl. Samples of 25 μ l of the indicated concentration were applied to the agar and scored after the indicated time interval had elapsed after the nematodes were added.

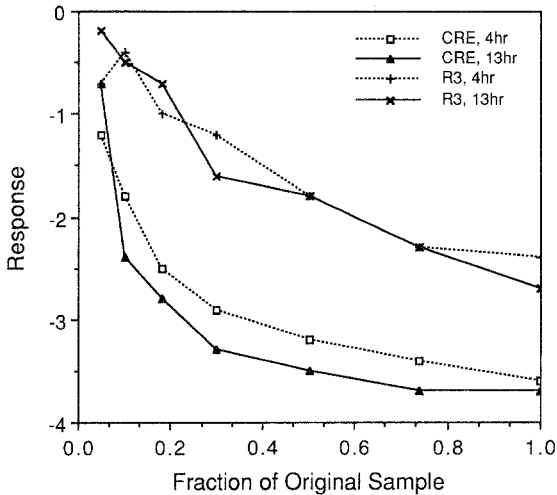


FIG. 2. Dose-response relationships for root exudate. Data for concentrated root exudate (CRE), and the major Sephadex peak (R3) are presented for nematode distributions 4 and 13 hr after nematode placement on the agar.

3.8 × 7.5 × 46 cm, made (on all six sides) from 2.5-cm-thick plastic foam. Three of these insulated boxes were then placed inside a larger insulated box, made from the same material but with internal dimensions of 15 × 28 × 53 cm. These elaborate procedures were taken to minimize thermal gradients to which these nematodes are extremely sensitive (Dusenbery, 1988c; Pline et al., 1988).

The distribution of nematodes on the agar within each box was observed under a stereomicroscope two or three times during the next 25 hr. The distribution was scored on the basis of a scale on which 0 corresponded to a symmetrical distribution around the center starting position, +5 corresponded to complete movement toward the sample, and -5 corresponded to complete movement away from the sample. Responses greater than four were rarely observed. This procedure was adopted after initial tests in which nematodes were counted in different sections of each box (Pline et al., 1988; Diez and Dusenbery, 1988) demonstrated that it was more useful to spend the additional time required to count nematodes on doing more replicates. In general, three replicates were performed for each sample tested, and the scores averaged together. The geometry of this test was chosen in order to make the assay equally sensitive to repellents and attractants.

Root Exudate. For a typical preparation of root exudate, eight tomato plants (Rutgers variety) that had grown about 30 days in a greenhouse after transplanting to 15-cm pots were washed free of soil. The roots were soaked in 2.0 liters of distilled water for 24 hr, while the tops were exposed to an artificial daylight regime. Approximately 1.5 liters of solution was recovered, and it was filter sterilized and refrigerated. In most cases, this raw root exudate was concentrated about 40-fold by rotary evaporation under reduced pressure at about 39°C. The resulting solution (concentrated root exudate) was frozen in small samples, if not used immediately. A variety of other methods of preparation were explored and most yielded similar results.

Solid-Phase Extraction. Residue extraction was performed by drying down 1-ml samples in glass test tubes, adding 2 ml of solvent, and vortexing. The solution was then transferred to a clean tube and the solvent evaporated. The residue from the second tube was redissolved in 0.5 ml water and tested for nematode responses.

Extraction from polar adsorbents was performed by drying down samples that were subsequently dissolved in 4 ml *n*-propanol. An equal volume of hexane was then added. The extraction was carried out in Baker 10SPE disposable columns containing silica gel or amino adsorbents, which were conditioned with propanol-hexane, 1:1. A sample of 0.2 ml was applied to each column. The columns were eluted with two 0.5-ml aliquots of each of several solvents of increasing eluting solvent strength (ϵ°) on silica (Baker-10SPE Application

Guide, vol. II, p. 112), and the effluent collected. Residual solvent was blown out between aliquots. Samples were dried down and resuspended in water for testing nematode responses.

Control samples were carried through each procedure in parallel with the samples being studied. This tested for artifacts caused by contaminants in the solvents or solid adsorbents.

Gel-Exclusion Chromatography. In a variety of experiments, concentrated root exudate was fractionated on Sephadex G-15 and G-50 and Biogel P2 and P4. Sephadex G-15 gave the best separations. The column used in the data presented here was 2.5 cm in diameter with a void volume of 85 ml and bed volume of 183 ml. The flow rate was 0.65 ml/min and fractions were collected at 6-min intervals. Ultraviolet light absorbance was measured at 280 nm with a flow-through detector. Fractions 92–96 from the run presented were pooled to make a sample called R3. The column was calibrated with molecular weight standards NaN_3 , reduced glutathione, glutathione disulfide, Evans blue, bacitracin, and blue dextran.

HPLC. A 25- μl sample of R3 was injected onto a C18 column (HP Hyperell ODS 5 μm , 100×2.1 mm, void volume 0.2 ml), which was eluted with 0.3 ml/min water (HP 1090 chromatograph). The effluent passed through a diode array detector (200–600 nm), and as drops emerged from the outlet, they were collected directly on the agar used to assay nematode responses. A similar procedure was also used with C8 and cyano columns.

RESULTS

In order to establish the sensitivity of our assay system, it was tested with NaCl, which is known to repel root-knot nematodes (Prot, 1980). Figure 1 shows the results of a test in which a 25- μl sample of various concentrations of NaCl were used in the assay. The results of observations at 4, 11, and 25 hr after the nematodes were placed on the agar are shown. An avoidance response is clearly apparent even at the earliest time with sufficiently high concentrations. Longer times clearly produced a more sensitive test with both significant responses at lower concentrations and stronger responses to high concentrations. However, there was little difference in sensitivity between 11 and 25 hr.

When the assay was applied to root exudates, the only strong or reproducible responses were avoidance (Table 1). This result was observed for exudates collected from a variety of plants (tomato, pepper, squash, watermelon, and soybean). Tomato was used as the source for subsequent studies. Rotary evaporation could be used to concentrate the activity and produce stronger responses that were easier to work with (Table 1). The volatile fraction contained no

TABLE 1. TYPICAL RESPONSES^a

Sample	Response			95% confidence interval
	Mean	SD	<i>N</i>	
Control	-0.1	0.2	8	-0.3 to 0.1
Raw	-1.4	0.6	15	-1.7 to -1.1
Concentrated	-3.6	0.4	5	-4.1 to -3.1
Volatile	-0.3	0.4	2	

^a "Raw" is the starting root exudate that was subjected to rotary evaporation to yield the "concentrated" and "volatile" fractions. *N* is the number of preparations of root exudate that were tested. "Control" is for parallel experiments using water.

activity (Table 1). The activity of the nonvolatile fraction was stable and could be boiled or dried down without appreciable loss. All the activity appeared to be polar based on extraction between aqueous and organic phases.

In a series of preliminary experiments, activity appeared to be retained by cation exchangers and not by anion exchangers. However, interpretation was complicated by the apparent production of stimulus activity by the ion-exchange resins. Further exploration indicated that varying the pH of samples between 4.5 and 9.5 did not affect the response to them.

These observations led us to try fractionating concentrated root exudate via gel exclusion chromatography. Testing a variety of gels indicated that Sephadex G-15 worked best. A typical result is shown in Figure 3. The basic pattern is a strongly repellent peak (running with an apparent molecular weight of about 1000 daltons on a calibrated column), followed by a well-separated weaker peak (running at about 500 daltons). Frequently, there was an indication of a third peak running between these two. This pattern was very reproducible. Of seven such chromatographic runs with different root exudate preparations, the one with the greatest departure from this pattern had one strong peak followed by hints of one or two slower peaks, and five runs had very much the same pattern. Fractions from the stronger, faster peak were pooled to form a sample (R3) for further study.

The polarity of R3 was explored by extracting from a solid phase with solvents of differing polarity (Figure 4). If samples of R3 are simply dried and extracted with a variety of solvents, methylene chloride and ethyl ether extracted little activity, while propanol, methanol, and water extracted nearly all. Acetone appeared to extract some, but not all, of the activity. If silica gel was used as an adsorbent, approximately the same polarity was necessary to solubilize the activity. If an amine adsorbent was used, somewhat more polar solvents

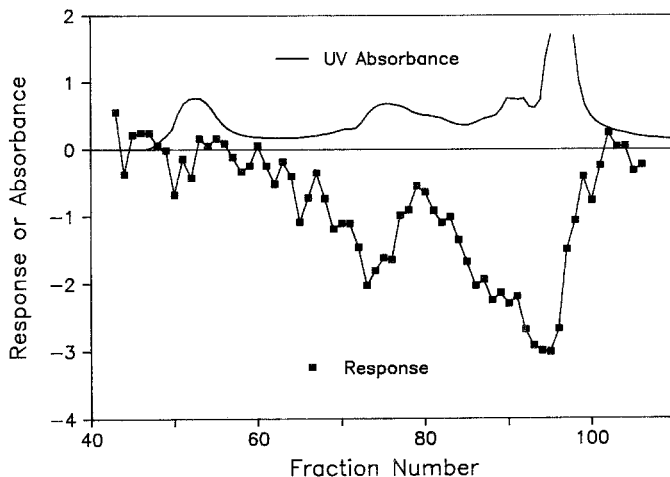


FIG. 3. Response to concentrated root exudate fractionated on Sephadex G-15. UV absorbance was measured at 280 nm. The large peak at fraction 95 is near the position expected for a typical molecule of 1000 daltons. The smaller peak near fraction 75 is near the position expected for a molecule of 500 daltons.

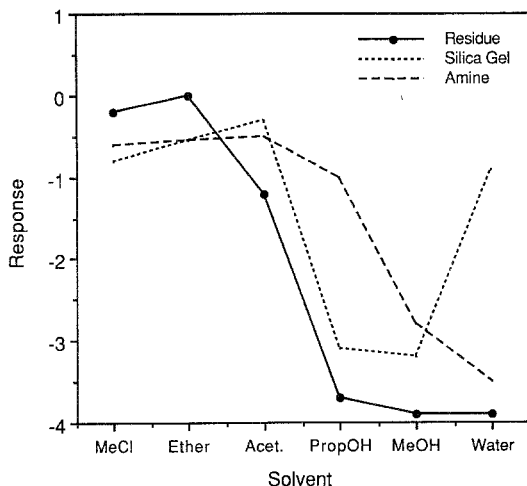


FIG. 4. Extraction of R3 samples with solvents of various polarities. The solid line is extraction of the dried residue with different solvents applied to different samples. The broken lines are successive extractions of solid-phase adsorbents with solvents of increasing eluting strength (ϵ°) on silica. Thus, in the case of silica gel, activity presumably declines with water because the active material has already been eluted from the adsorbent.

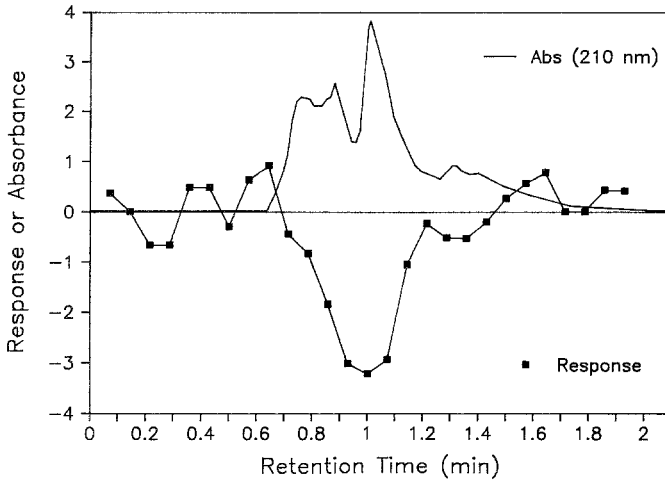


FIG. 5. Response to R3 fractionated on an HPLC C18 column. Eluted with water. Absorbance is reported for 210 nm.

were necessary. Activity in R3 did not bind appreciably to C18 or cyano non-polar adsorbents.

In order to fractionate R3 further, experiments were begun to exploit HPLC techniques. A standard C18 column with water elution was used initially and drops of effluent collected directly on the agar surface of an assay box. The results (Figure 5) demonstrate that this approach is practical; a clearly defined peak of activity is observed. The peak appears quite broad, but this appearance may be an artifact of nonlinearity of the assay. As seen in Figures 1 and 2, the response is nearly proportional to the log of the concentration. If this relation is used to estimate the concentration of stimulus in Figure 5, the activity peak is substantially narrower. There is no clear correspondence between activity and UV absorbance. Similar behavior was observed with C8 and cyano columns.

DISCUSSION

The results reported here demonstrate that one can reliably measure a chemotactic response and use it as a bioassay to follow repellent activity through a variety of chemical fractionation procedures. The assay used is sufficiently sensitive to detect the response to $10 \mu\text{g NaCl}$, which produces an average concentration of $44 \mu\text{M}$ in the agar of the assay.

The fractionation experiments demonstrate clearly that the repellent is a polar molecule. Chromatography on Sephadex indicates it is of medium size

and that there are at least two different-sized molecules involved. The experiments initiated with HPLC demonstrate that the assay technique can be used successfully with HPLC and this approach probably holds the most promise for purification of the active chemicals.

An important question to address is whether the repellent characterized here actually came from the roots. One possibility (although far-fetched) is that the repellent was a contaminant of the deionized distilled water in which the roots were soaked. This possibility is eliminated by several contrary observations: (1) testing water without a control amount at the other end of the assay box led to attractive responses, which we attribute to a response to water activity (Prot, 1980); (2) incubation with roots changed this attraction to repulsion; (3) concentration of water samples fivefold and testing in the standard way produced responses not significantly different from water controls. Another possibility is that the repellent is produced by microorganisms associated with the roots rather than the roots themselves. We have not attempted to distinguish between these two possibilities. When we speak of root exudate, we mean a product of the rhizosphere, not necessarily a direct product of the plant.

Considering that we are looking at the product of millions of years of evolution in which nematodes have been selected for more efficient ways of locating hosts and the plants have been selected to avoid nematodes, it might be anticipated that a complex of repellent and attractant chemicals would be found to influence the interaction between host and parasite. Although it was suggested over 30 years ago that host roots have repellents as well as attractants (Wieser, 1956), this has remained a controversial point (Croll, 1970; Prot, 1980). Probably the most surprising finding of this study is that root exudate appeared to contain only repellent activity. Extensive efforts to uncover attractant activity by fractionation and other manipulations of the exudate were not successful in revealing attractant activity that was strong or reproducible. The repellent is obviously of potential benefit to the host; it could also benefit the nematode by directing it to appropriate sites for entry. This could occur, for instance, if mature roots, as opposed to growing root tips, are the source of the repellent. Resolution of these possibilities must await detailed studies of the site of release and the distribution of the chemicals around the rhizosphere.

It must be remembered that the assay used here is not expected to work with highly volatile stimuli and that there are several previous observations demonstrating that these and other plant-parasitic nematodes are attracted to carbon dioxide (Klingler, 1963; Pline and Dusenbery, 1987; Prot, 1980). These results suggest that carbon dioxide is the principle means by which nematodes locate host roots. As a cue, CO₂ has the advantage that it would be difficult if not impossible for a plant to stop releasing it under the selection pressure of nematode parasites. On the other hand, it has the disadvantage that it is not specific. There are many sources of CO₂ in soil, although most are at least

associated with roots. The lack of more specific attractants in the case of root-knot nematodes may be related to the fact that they are generalists with a very broad host range. It would be interesting to determine if nematodes with a narrower host range, such as cyst nematodes, utilize more specific cues. In summary, our current conclusions are that CO₂ is probably the primary stimulus by which root-knot nematodes locate hosts and that the repellent is either too weak to be effective or is released at a different site in the rhizosphere.

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(Z,Z)-7,9-DODECADIENYL ACETATE, SEX
PHEROMONE OF *Epinotia tedella* CLERCK
(LEPIDOPTERA: TORTRICIDAE)

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Abstract—(Z,Z)-7,9-dodecadienyl acetate, a reported male attractant for several New World spp. of *Epinotia*, was identified as the primary pheromone of female *E. tedella* (European spruce budmoth) through chemical analysis of pheromone gland washes, the electrophysiological study of male antennal receptor types, and field-trapping tests. The washes contained this compound at a level of approximately 0.4 ng per FE, along with a similar amount of the corresponding alcohol, (Z,Z)-7,9-dodecadien-1-ol. Each compound activated its own specialized type of male receptor cell. No traces of stereoisomers or monoenes were found in the washes. In field-trapping tests conducted in stands of Norway spruce in southern Germany, (Z,Z)-7,9-dodecadienyl acetate as a single chemical proved highly attractive to male *E. tedella* over a range of lure doses. The corresponding alcohol or aldehyde did not show attractivity; rather, in binary combinations with the primary pheromone, these compounds, and also the (E,Z)-7,9 stereoisomeric acetate, reduced captures. A 10:1 blend of (E)-9- and (Z)-9-dodecenyl acetates, reported as an *E. tedella* male attractant, did not reveal significant captures. No other *Epinotia* spp. besides *E. tedella* responded to the various 7,9-dodecadienyl test baits during this study.

Key Words—(Z,Z)-7,9-Dodecadienyl acetate, pheromone, sex attractant, attraction inhibitors, olfactory receptors, *Epinotia tedella*, *Epinotia* spp., Tortricidae, Lepidoptera.

INTRODUCTION

The spruce budmoth *Epinotia tedella* Clerck (Tortricidae: Eucosmini) is the most common microlepidopteran infesting Norway spruce, *Picea abies* (L.) Karst, in Europe and its impact on growing rates of trees is well documented (Führer, 1978). Usually the losses caused by this moth have not prompted control measures, even at high infestation levels. This view is changing as a result of the severe dieback of central European spruce forests due to airborne emissions, which appears to have increased their vulnerability to attack by secondary insect pests (Führer, 1985; Sierpinski, 1985; Baltensweiler, 1987). The monitoring of population fluctuations of forest insect species hitherto considered neglectable, such as *E. tedella*, is thus now timely.

The present study was initiated in order to identify the chemical composition of the *E. tedella* female pheromone and to develop a standard male attractant appropriate for routine population monitoring. While this study was being carried out, Booij and Voerman (1984) reported male *E. tedella* captures by a mixture of (*E*)-9- and (*Z*)-9-dodecenyl acetates, whereas Reed and Chisholm (1985) demonstrated specific attractiveness of certain 7,9-dodecadienyl compounds for North American species of the genus *Epinotia*.

METHODS AND MATERIALS

Insects. The *E. tedella* moths used in the chemical and electrophysiological analyses were collected as mature larvae, from spruce trees near Seewiesen, southern Germany, in late autumn. The larvae were provided sand for pupation and the pupae kept in outdoor conditions until early spring. Sexed pupae and adults were held in a 16:8 hr light-dark photoperiod at room temperature.

Test Chemicals. The dodecadienyl alcohols, acetates, and aldehydes were synthesized according to the methods described by Reed and Chisholm (1985) and had a purity of >98% and a steric purity of >99% by GC analysis. The monoenic analogs included in the field tests were purchased from the Institute for Pesticide Research, Wageningen, the Netherlands, in a steric purity of >99%. The series of olefinic compounds used in the electrophysiological tests were from our laboratory collections.

Electrophysiology. Electroantennograms (EAGs) were recorded from excised male antennae, whereas nerve impulse responses of single receptor cells were monitored from the cut ends of sensilla trichodea. Recording, stimulation, and data evaluation were as in studies on other tortricid moths (Priesner, 1983).

Pheromone Gland Washes. These were made from 1- to 3-day-old females at 2-3 hr after the beginning of scotophase. The extruded abdominal tips of 102

unmated females were dipped singly into *n*-hexane (Merck, for residue analysis; 3 μ l per female) for 5–10 sec and the wash stored at -18°C until use.

Chemical Pheromone Analysis. The analysis of pheromone present in the gland washes was similar to the method used by Priesner et al. (1984). Capillary gas chromatography (GC) and coupled GC–mass spectrometry (GC-MS) were carried out on samples of 20 FE per analysis, concentrated to 2 μ l. Further methodological details will be presented where relevant in the results section.

Field-Trapping Procedures and Study Sites. Synthetic baits were tested for attractancy to male *E. tedella* using tetratraps (Arn et al., 1979; sticky surface, 145 cm²) and rubber cap lure dispensers (red serum bottle caps, diameter 18 mm). The caps were impregnated with chemicals from dilutions in *n*-hexane and approx. 10 mg of antioxidant (2,6-di-*tert*-butyl-4-methylphenol) was added routinely. Lure sources of different chemical composition were compared as replicated series each comprising up to 14 traps. These were suspended, in a randomized order, from spruce branches 1.5–2 m off the ground, at distances of 2–5 m within series and 50 m or more between replicates. The traps were inspected once or twice weekly, collecting specimens and replacing the sticky liners where necessary. Trapping data from the replicated series were transformed to $\log(x + 1)$ and submitted to an analysis of variance.

Additional tests were aimed at assessing the effects of lure dose and trap placement on capture rates. A varying amount (0.1–1000 μ g) and a standard amount (100 μ g) of (*Z,Z*)-7,9-dodecadienyl acetate, respectively, were used in these tests.

Spruce forests of different age classes, representing varying levels of *E. tedella* infestation, were included in the study. The edges of older stands, showing a more homogeneous infestation by the moth, were generally used for placing the specificity and dosage series. The main sites were located near Freiburg and Mengen, Black Forest, and near Seewiesen, Upper Bavaria.

RESULTS

Electroantennography. Analysis of the *E. tedella* pheromone system began by recording male EAG responses to identified tortricid pheromone components and various structural analogs. First, an overall screening of test compounds (acetates, alcohols, and aldehydes, differing in chain length and double-bond position) was carried out at the standard amount of 1 μ g. These tests revealed (*Z,Z*)-7,9-dodecadienyl acetate (*Z7,Z9*-12:Ac) as the compound evoking the strongest EAG response. Relatively large responses were also recorded for the corresponding alcohol and aldehyde analogs, *Z7,Z9*-12:OH and *Z7,Z9*-12:Ald, and the stereoisomeric and 9-monoenic acetates, *Z7,E9*-12:Ac,

E7,Z9-12:Ac, *Z9-12:Ac*, and *E9-12:Ac*. Subsequent comparison of EAG dose-response curves showed that these six compounds were between 3- and 30-fold less stimulatory than *Z7,Z9-12:Ac*. Other acetates, alcohols, and aldehydes tested were at least 100-fold less stimulatory.

Single Receptor Study. By recording nerve impulse patterns from the cut tips of male antennal sensilla trichodea, two prevailing cell types were defined. The one was an acetate receptor maximally responsive to *Z7,Z9-12:Ac*, and the other, an alcohol receptor specific to *Z7,Z9-12:OH*. Most recordings showed action potentials originating from both types of cell, partially separable by spike amplitude and selective cross-adaptation. No efforts were made to define further potential cell types.

Chemical Pheromone Identification. Components with similar retention times to authentic *Z7,Z9-12:OH* and *Z7,Z9-12:Ac* were detected, in the gland washes, by flame ionization GC using a Hewlett Packard model 5890 gas chromatograph equipped with a DB-1 fused silica capillary column (30 m \times 0.3 mm ID; J&W Scientific Inc., Rancho Cordova, California) temperature programmed from 100°C to 250°C at 4°/min.

Identities of these components were verified by chemical ionization GC-MS using methane as the reagent gas and hydrogen as the carrier gas on a Finnigan model 3300 mass spectrometer coupled to an Incos model 2300 data acquisition system. Analyses were made using undecane and tetradecane as internal standards. The samples were chromatographed on a DB-5 capillary column (60 m \times 0.35 mm ID; J&W Scientific) under similar temperature program conditions. Standard 7,9-dodecadienyl aldehyde, alcohol, and acetate isomers eluted in the order: *ZE*, *EZ*, *ZZ*, *EE*. In the tip washes, two compounds were found whose retention times and mass spectra coincided with authentic standards of *Z7,Z9-12:OH* and *Z7,Z9-12:Ac*. Evidence included the diagnostic ions $[M - 1]^+$, $[M]^+$, and $[M + 1]^+$ for both compounds; $[(M + 1) - 18]^+$ for *Z7,Z9-12:OH*, and $[(M + 1) - 60]^+$ for *Z7,Z9-12:Ac*. No aldehydes or other alcohol and acetate isomers could be detected.

The compounds were quantitated by comparing their total ion counts in the extract to an external calibration curve. The alcohol and acetate were thus estimated at levels of 0.4 ng/FE each.

Field Trapping. *Z7,Z9-12:Ac* as a single compound proved strongly attractive to male *E. tedella*. On testing different lure doses (data not specified), *Z7,Z9-12:Ac* sources ranging from 0.1 to 1000 μ g revealed captures, which generally increased with increasing lure dose. The alcohol, *Z7,Z9-12:OH*, did not produce significant captures.

Potential modifying effects of second components were studied by means of binary combinations made with 100 μ g of *Z7,Z9-12:Ac*. As a 10- μ g addition (Table 1, series 1), the *EZ* stereoisomer strongly reduced captures; the *ZE* stereoisomer and the *ZZ* alcohol and aldehyde appeared to cause some reduc-

TABLE 1. CAPTURES OF MALE *Epinotia tedella* IN TETRATRAPS BAITED WITH 7,9-DODECADIEINES AND 9-MONOENES.^a

Lure composition (μg)	Total catch	
	Series 1	Series 2
Z7, Z9-12: Ac		
10	59 ab	34 b
100	123 a	101 a
100 + Z7, Z9-12: OH		
10	36 b	
100		9 c
100 + Z7, Z9-12: Ald		
10	47 b	
100		14 c
100 + Z7, E9-12: Ac		
10	58 ab	
100		132 a
100 + E7, Z9-12: Ac		
10	8 c	17 bc
100		2 c
100 + E7, E9-12: Ac		
10	111 a	
100		114 a
100 + E9-12: Ac		
10	109 a	
100		135 a
100 + Z9-12: Ac		
10	109 a	
100		99 a
Z7, Z9-12: OH (100)		0 c
Z7, Z9-12: Ald (100)		0 c
E9-12: Ac (100) + Z9-12: Ac (10)	14 c	15 c
Blank		8 c

^aMengen, June 10/13 to July 14/18, 1986 (series 1) and Seewiesen, June 19 to July 29, 1987 (series 2). Each series four replicates. Numbers within the same column followed by the same letter are not significantly different ($P = 0.05$, Tukey's test).

tion, whereas the *EE* stereoisomeric and the Z9 and E9 monoenic acetates had no apparent effect. These same seven compounds were subsequently tested as 100- μg additions. The results (Table 1, series 2) confirm the strong inhibitory properties for the E7,Z9-12: Ac, and show that the Z7,Z9-12: OH and Z7,Z9-12: Ald are also inhibitors, whereas the four others appear to have virtually no effect on captures.

Traps baited with the 10:1 mixture of E9-12: Ac/Z9-12: Ac, the *E. ted-*

ella attractant reported by Booij and Voerman (1984), were included in both series. This mixture revealed only poor captures, not significantly differing from blank traps (Table 1). The Z7,Z9-12:OH and Z7,Z9-12:Ald as single compounds did not catch even a single male *E. tedella*.

No other species of *Epinotia* besides *E. tedella* were captured during this study.

DISCUSSION

The results of the electrophysiological, chemical, and field studies identify Z7,Z9-12:Ac as the primary female pheromone of *E. tedella* and as a highly potent male attractant.

No evidence for pheromone synergism was found. In the field tests, the alcohol, Z7,Z9-12:OH, although present in the pheromone gland washes at almost the same amount as the acetate, did not show attractivity as a single chemical and reduced responses to the acetate when added to it in amounts of 10% or more. Whether this alcohol is actually released by calling *E. tedella* females, and whether it has potential pheromonal effects in amounts lower than 10%, remains unknown. The three stereoisomeric acetates, and also the 7- and 9-monoenes, were not found in the washes at levels of >1% of the two major components (detection limit) and probably are not part of the pheromone.

Three different compounds, E7,Z9-12:Ac, Z7,Z9-12:OH, and Z7,Z9-12:Ald, acted inhibitorily on trap captures. These data suggest that the *E. tedella* male receptor system includes, besides the Z7,Z9-12:Ac and Z7,Z9-12:OH receptors found in the electrophysiological recordings, specialist cells also for the EZ pheromone stereoisomer and for the ZZ aldehyde analog.

The results of the present study indicate a close pheromonal relationship of *E. tedella* to three New World *Epinotia* spp. studied by Reed and Chisholm (1985): *E. silvertoniensis* (Kft.), *zandana* (Kft.), and *removana* McDunnough (the last two referred to by the authors as species A and B, respectively). The pheromone of these three species have not yet been chemically identified, but in the field the males of these species were strongly attracted by Z7,Z9-12:Ac, as with *E. tedella*. The alcohol, Z7,Z9-12:OH, was another potent attractant for *E. removana* but inhibitory on *E. silvertoniensis*. The E7,Z9-12:Ac and the monoene Z9-12:Ac both inhibited attraction of male *E. zandana* but had little effect on the two other species. Together with the *E. tedella* data reported here, these differential response patterns suggest a role of secondary pheromone components for maintaining reproductive isolation among species of *Epinotia* which share Z7,Z9-12:Ac as their primary female pheromone.

Booij and Voerman (1984) described a 10:1 blend of E9-12:Ac/Z9-12:Ac as an *E. tedella* male attractant, based on captures of 409 males of this species

recorded in a single trap containing this mixture. In our replicated series, this mixture failed to produce significant *E. tedella* captures. The reason for our failure to reproduce the above result is unknown.

It is noteworthy that, during this study, no other species of *Epinotia* besides *E. tedella* were captured with Z7,Z9-12:Ac (alone or in mixtures). The genus is represented in central Europe by some 35 species (Hannemann, 1961), some of which co-occur on Norway spruce together with *E. tedella*. For one of these, *E. nanana* (Treits.), a 1:1 blend of (*E*)-8- and (*E*)-10-dodecenyl acetates has been reported as the specific male attractant (Booij and Voerman, 1984).

Our results show that 100 μ g of Z7,Z9-12:Ac can be used as a standard trap bait for monitoring *E. tedella* populations, without need for improving, by secondary bait components, capture efficiency or selectivity. Isomerization of the Z7,Z9-12:Ac during field exposure has not yet been determined, but it is assumed to be similar to (Z, Z)-8,10-dodecadienyl acetate (Chisholm et al., 1985). This isomerization may be critical to the lure's relative attractancy over time if significant amounts of the E7,Z9-12:Ac are produced.

In 1986, a program using this standard attractant was started with the aim to evaluate trap position effects and to correlate moth captures with local population densities based on larval counts. Results of this aspect of the study will be reported elsewhere.

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VOLATILES RELEASED FROM INDIVIDUAL SPRUCE BARK BEETLE¹ ENTRANCE HOLES Quantitative Variations During the First Week of Attack

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Abstract—Volatiles released from individual entrance holes of eight spruce bark beetles (*Ips typographus*) were collected during the first week of attack on a resistant host tree. In order to quantify the release of the highly volatile 2-methyl-3-buten-2-ol (MB) from attacking males, a new method was developed with deuterated quantification standard released at the time of collection. The amounts of collected volatiles, as analyzed by GC and GC-MS, showed a large variation between individual holes and also between subsequent entrainments from the same hole. Most of the quantified compounds on the average have two maxima, with a pronounced intervening depression. The amounts of released *cis*-verbenol (cV) increased five times during the first two days, while the amounts of MB were consistently high. The attacked spruce tree was not taken by the beetles, and the average amounts of the two aggregation pheromone components, MB and cV, increased again after the first maxima. The first peak of oxygenated monoterpene, released in the beginning of the attack containing α -terpineol, terpinen-4-ol, bornyl acetate, *trans*-pinocarveol, and verbenone, was possibly due to spontaneous oxidation of monoterpene hydrocarbons from the tree. Microorganisms established in the gallery wall phloem probably participated in the production of oxygenated monoterpenes during the second increase.

Key Words—*Ips typographus*, Coleoptera, Scolytidae, *Picea abies*, aggregation pheromones, host tree resistance, tree-switching, GC-MS, monoterpenes, 2-methyl-3-buten-2-ol, *cis*-verbenol, verbenone.

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INTRODUCTION

Several species of bark beetles are known to produce aggregation pheromones when attacking their host trees (Vité and Francke, 1976; Borden, 1982; Wood, 1982). The pheromone of each species is usually a mixture of compounds. The behavioral activity of the individual compounds varies and may also elicit different steps in the behavior chain (Borden, 1974; Dickens, 1981; Schlyter et al., 1987a,b). In *Ips*, the production of pheromone components, reflected as their content in bark beetle hindguts, has been shown to start as soon as the males bore into the host tree phloem (Birgersson et al., 1984). It reaches a maximum during excavation of the nuptial chamber, and decreases when the males admit females, mate, and the females excavate galleries and lay eggs (Byers, 1981a; Birgersson et al., 1984).

The defensive monoterpene hydrocarbons produced by the host tree also show a quantitative variation as the attack of the spruce bark beetle (SBB, *Ips typographus* L.) proceeds (Leufvén and Birgersson, 1987). However, there is a marked difference in the quantitative patterns between the host-tree-produced compounds and the bark-beetle-produced ones. The amount of monoterpene hydrocarbons from the tree, after an intervening decrease, increases again during the end of the first week of attack, while the amount of volatiles found in bark beetle hindguts decreases continuously after a first rapid increase, when the males attack the spruce tree (Leufvén and Birgersson, 1987; Birgersson and Leufvén, 1988). Oxygenated monoterpenes present in the gallery wall phloem also show a quantitative variation during an attack (Leufvén and Birgersson, 1987). The amount of α -terpineol, borneol, terpinen-4-ol, and verbenone increased while the females elongated their galleries, especially when the gallery walls were brown-stained. This coloring is probably a sign of established microorganism.

In a theoretical model, Schlyter and Anderbrant (1989) have compared the quantitative variation of hindgut volatiles in male SBB attacking standing spruce trees (Birgersson *et al.*, 1984) with the dynamics of naturally attacking SBBs. This numerical model attempted to describe the process leading to a mass attack with regard to the fast initial production of the aggregation pheromone components 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol (cV). However, the model failed to describe the chemical events accompanying the shifting from attacked and taken host tree, known as "tree-switching" (Gara and Coster, 1968); their predicted decrease of attractive semiochemicals comes too late. In addition, the production of ipsenol (Ie), with known antiaggregative properties (Bakke, 1981; Schlyter et al., 1989), is too low and also appears too late, if at all. Probably, there are missing components in the tree-switching phenomenon; some of these compounds may not be produced by the bark beetles themselves but are released

from the phloem surrounding the galleries where microorganisms have been established.

The behaviorally active compounds are not accurately represented by the quantities in bark beetle hindguts or the quantities in gallery wall phloem, but only by the amounts actually released from the bark beetle entrance holes. Most of the odor/volatile entrainments made from bark beetles have been done on batches of beetles excavating in cut logs (Young et al., 1973; Browne et al., 1979; Schlyter et al., 1987a). For nonaggressive species, which naturally attack dead material, such as windthrown trees and forest debris, laboratory studies can and/or may indeed show which compounds these beetles produce during their attacks. However, for some aggressive species, which are able to attack living trees and utilize host precursors in pheromone production, such as *I. typographus*, log experiments are not appropriate. A log is not able to mobilize a resin defense, neither by the primary nor by the secondary increase of toxic monoterpene hydrocarbons, as is a living tree (cf., Leufvén and Birgersson, 1987). In this regard, males of SBB excavating in spruce logs in the laboratory produce very small amounts of pinene alcohols (i.e., cV, *trans*-verbenol (tV), myrtenol (Mt), and *trans*-myrtenol (tM)) (Birgersson, unpublished).

Males of *I. typographus* are known to show a rapid increase in their production of aggregation pheromone components when they bore into a standing host tree (Birgersson et al., 1984). But exactly how soon after their attack do they release their semiochemicals to attract conspecific males and females to overcome the resistance of the selected host tree? We know that the variation in hindgut content of volatiles is very large, even between males in the same attack phase and attacking the same host tree (Birgersson et al., 1988).

In the present study we have quantified the amounts of different volatiles released from individual SBB entrance holes during the first week of an attack. We describe the method that was used and the quantity of the airborne aggregation pheromone components from attacking males as well as monoterpene hydrocarbons and oxygenated monoterpenes from the injured phloem. Since the entrainments were done from individual entrance holes, the results can be related to the attack success of each male.

METHODS AND MATERIALS

Biological Material and Sample Collection. The field work was carried out in Gribskov, 10 km north of Hillerød, Sjaelland, Denmark, during the main swarming period of *I. typographus* in late May 1985. The number of swarming SBB was low, and to ensure attack and colonization the selected Norway spruce tree [*Picea abies* (L.) Karst.] was baited with a commercial pheromone attrac-

tant for *I. typographus* (Ipslure, Borregaard a/s, Norway). Compounds volatilizing from each of eight entrance holes of SBB males, attacking the same spruce tree, were collected in 3-hr periods for one week. The selected spruce tree, with a diameter at breast height of 22.5 cm, was standing on the forest edge, which opened to the southwest. The tree was characterized as being in good physiological condition, indicated by many fresh spring shoots and a rich resin flow from hand-made holes in the bark. After attack by SBB, the tree was not judged as taken by the beetles, mainly due to the low number of swarming beetles and very few attacks on the tree (≤ 1 male/dm²). All the sampled entrances were located between 1 and 2 m above ground. The males attacked the tree during the early afternoon, and the first set of entrainments was started at 1630 hr. During the first 27 hr of the attack, the entrainment was continuous, and thereafter entrainments were done each afternoon (from 1400 to 1700 hr), when the production in pilot studies during the previous years was found to be largest.

During the entrainments, a silanized glass T (Figure 1) was attached outside each entrance hole, and stapled to the tree using a piece of rubber tubing. The volatiles were trapped on synthetic polymer adsorbent plugs; PTFE tubings

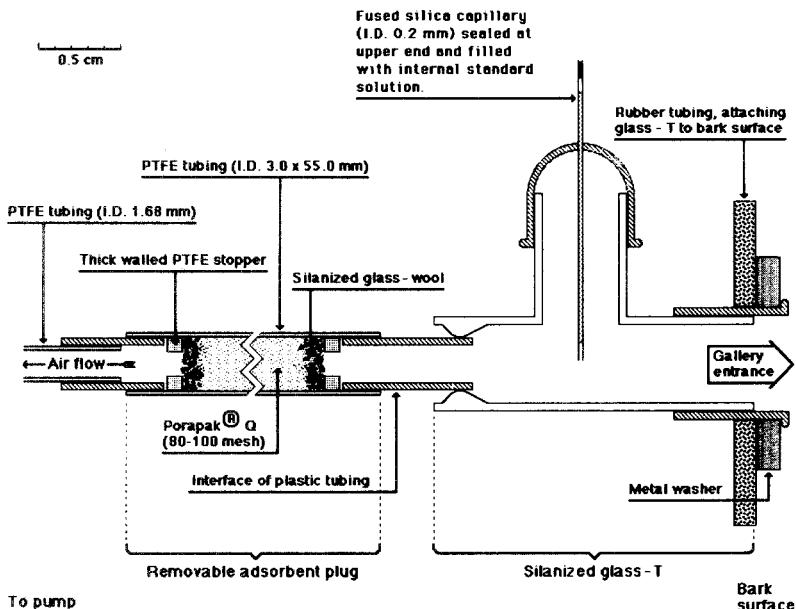


FIG. 1. Glass T used in entrainments from individual entrance holes. Internal standards (deuterated 2-methyl-3-buten-2-ol and pentanol dissolved in undecane, 1:1:8) are released from fused silica capillaries, one end sealed with beeswax.

(ID 3 mm × 55 mm) filled with Porapak Q (70 mg; 80–100 mesh). Pilot studies had shown that these plugs quantitatively adsorbed ($\geq 99\%$) all compounds released from bark beetle entrance holes, except the highly volatile 2-methyl-3-buten-2-ol (MB). In order to quantify the release of MB, internal standards were used at the time of collection. Deuterated-MB (D-MB) $[(D_3C)_2C(OH)CH=CH_2]$ and pentanol dissolved in undecane were released from a fused silica capillary (ID 0.2 mm × 25 mm), one end of which was sealed with beeswax (Figure 1). Pilot studies of the release of D-MB and pentanol showed these two compounds to have almost equal release rates, both when released separately from different capillaries and as a mixture dissolved in undecane (1 : 1 : 8). The latter was analyzed as the remains in the capillaries. In addition, pentanol is quantitatively adsorbed on the Porapak Q plugs used in this study. A small suction air-pump was used to achieve an air flow through each adsorbent plug of about 20 ml/min. Air entered between the glass T and the bark surface, just over and surrounding the entrance hole. Thus all volatiles released from the hole were drawn through the glass T and trapped on the adsorbent plugs. The glass T's were removed between each afternoon entrainment so that females could be admitted. All glass T's were washed in ethanol between the afternoon entrainments. The first set of entrainments was started only a few hours after the male beetles first attacked the spruce tree; i.e., as soon as it was possible to put the glass T's over the entrance holes without disturbing the excavating beetles. Some of the males were not yet completely into the phloem. The attack phase status of the beetles in each single attack after the entrainment period is shown in Table 1. The Porapak Q plugs were stored in liquid nitrogen until chemical analysis.

Chemical Analysis. The collected volatiles were extracted from the entrainment plugs with 400 μ l of *n*-pentane (doubly distilled). This volume of pentane proved enough to quantitatively elute the volatiles from the adsorbent plugs, since a second rinse with 400 μ l pentane gave less than 0.1% of monoterpene hydrocarbons and undetectable amounts of oxygenated compounds in pilot analyses. To enable GC and GC-MS quantifications of monoterpene hydrocarbons and oxygenated monoterpenes, respectively, 40 μ g tetradecane and 500 ng heptyl acetate (C_7Ac), dissolved in 100 μ l *n*-pentane (doubly distilled), were added to each entrainment extract.

Without prior concentration, the volatile extracts were analyzed on a Hewlett-Packard 5830A GC, using a fused silica capillary column (46 m × ID 0.35 mm) coated at our department with OV-351 (Ohio Valley) (Lanne et al., 1987). Nitrogen was used as carrier gas ($Q = 15$ cm/sec) and the temperature programming was 60°C for 4 min, 5°C/min to 210°C. The amounts of monoterpene hydrocarbons were quantified using the added standard, 40 μ g tetradecane, as described by Leufvén and Birgersson (1987).

TABLE 1. STATUS OF BARK BEETLES AT END OF 7-DAY ENTRAINMENT PERIOD

	Entrance hole number							
	1	2	3	4	5	6	7	8
Attack phase ^a	6	6	3	6	5	2 ^b	3	6
No. of females	2	2	2	2	2			2

^aAttack phase 2: unmated male fully in the phloem; attack phase 3: unmated male with nuptial chamber; attack phase 6: mated male with egg-laying females in 2- to 4-cm-long female galleries, as defined in Birgersson et al. (1984).

^bNo male at end of entrainment period.

After the GC analysis, the extracts were concentrated in tapered glass tubes on a lab bench at room temperature, according to Klimetzek et al. (1989) to about 10 μ l. Three microliters of the concentrated extract was analyzed by a Finnigan 4021 GC-MS (quadrupole) system, continuous scanning over the region m/z 29–300, with exception of m/z 32, with one scan per second. The GC was equipped with a fused silica capillary column (25 m \times ID 0.15 mm) coated at our department with Superox FA (Alltech) (Lanne et al., 1987). The temperature programming was 50°C for 4 min, followed by an increase of 8°C/min to 200°C, and isothermal at this temperature for 15 min. Helium was used as carrier gas ($Q = 25$ cm/sec).

Several compounds, mainly oxygenated monoterpenes identified in SBB hindguts (Birgersson et al., 1984, 1988) and in gallery wall phloem (Leufvén and Birgersson, 1987), were searched for and quantified, using "extracted ion current profiles" (EICP) (Chen, 1979; Garland and Powell, 1981). For each compound, three to four prominent and/or typical MS fragments were used and the calculated amounts were compared with the added standard, 500 ng heptyl acetate, as described by Birgersson et al. (1984) and Leufvén and Birgersson (1987). According to pilot studies, the limit of quantification (LOQ) was estimated to be 10 ng, and the accuracy of the quantification to be $\pm 5\%$. To quantify the highly volatile MB, the ratio between m/z 71 for MB and m/z 74 for D-MB was compared with the amount of pentanol, which was released together with D-MB at the time of collection. For MB, LOQ was estimated to be 100 ng, and the accuracy $\pm 5\%$.

Since the number of sampled individuals was low, no thorough statistical analysis was done. We employed various programs in the Statgraphics program package, produced by Statistical Graphics Corp., Princeton, New Jersey, to examine trends in the data.

RESULTS

The amounts of different volatiles released from the entrance holes were found to show a very large variation among SBB males attacking the same host tree. A large variation was also found between subsequent entrainments from the same entrance hole. The results are presented both as general trends and as variations among different entrance holes.

Host-Tree-Produced Compounds. From most of the entrance holes, very large amounts of monoterpene hydrocarbons were passively emitted, even during the first 3-hr entrainment (Figure 2). During the first evening and night, the amounts of monoterpene hydrocarbons, such as α -pinene, β -pinene, 3-carene, myrcene, limonene, and β -phellandrene, emitted from the host tree, decreased, probably due to lower temperature during the night (3°C at 0530 hr. = sunrise; Figure 3), the release increased during the morning and reached a maximum in the afternoon. There was a decrease in emitted amounts of monoterpene hydrocarbons between days 3 and 5, but the emitted amounts increased clearly again during the last days of the first week, days 5 to 7 (Figure 2A).

Release of monoterpene hydrocarbons from most of the individual attacks followed the general pattern shown in Figure 2B. Large amounts of monoterpene hydrocarbons were emitted from entrance hole 8 during the first days of the attack. The resin flow from this entrance was relatively high, since a pitch tube was formed around the entrance hole. After the general decrease during days 3–5 (Figure 2B), the emitted amounts of monoterpene hydrocarbons from this individual did not increase during the last days. From entrance hole 6, only

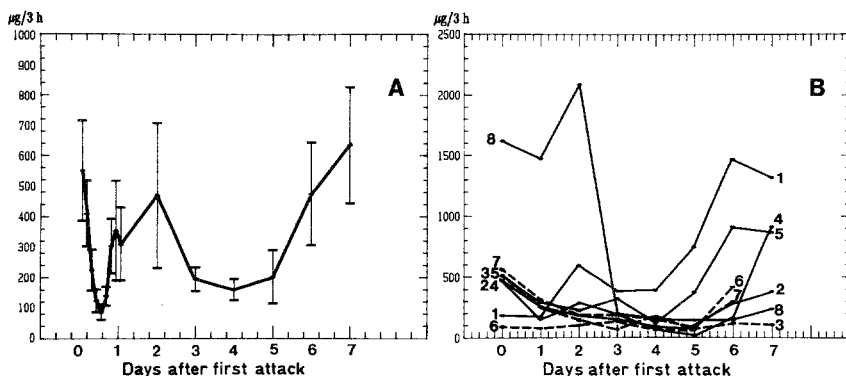


FIG. 2. Amounts ($\mu\text{g}/3\text{ hr}$) of total monoterpene hydrocarbons collected during the entrainment period. (A) Mean \pm SE. (B) Entrainments from individual entrance holes. -- unmated males, — mated males, respectively, at the end of entrainment period.

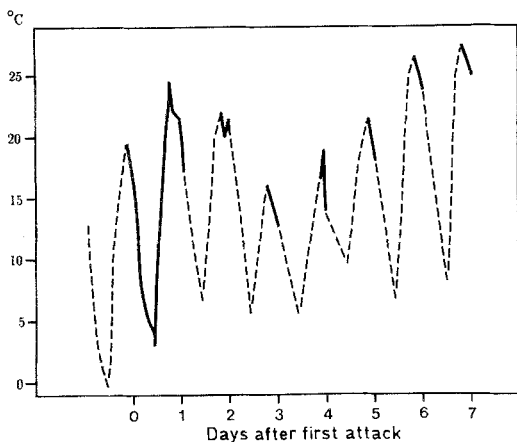


FIG. 3. Temperature in Gribskov during the entrainment period. Solid line during the entrainments.

small amounts of monoterpene hydrocarbons were emitted, probably due to the low excavating activity of the attacking male. At the end of the entrainment period, only a short tunnel was found in the phloem, and the male was gone.

Bark-Beetle-Produced Compounds. Eight compounds that have been identified in bark beetle hindguts (Birgersson et al. 1984), 2-methyl-3-buten-2-ol (MB), *cis*-verbenol (cV), *trans*-verbenol, myrtenol, *trans*-myrtenol, ipsdienol, ipsenol, and 2-phenylethanol, were searched for and quantified. The two essential aggregation pheromone components, MB and cV (Schlyter et al., 1987b), showed different general patterns of appearance. The general trend for MB was continuously increasing amounts during the entrainment period (Figure 4A). On the other hand, the general pattern for cV was a fast increase during the first and second day of the attack, followed by decreasing amounts (Figure 5A). The maximum amounts of MB released during a 3-hr entrainment was 65 μg (Figure 4B), which corresponds to 0.4% of the body weight (dry weight) and was produced by a male with two females in ≥ 2 -cm-long galleries (No. 4). The corresponding maximum of cV was almost 700 ng (Figure 5B). In general, the released amounts of *trans*-verbenol, myrtenol, *trans*-myrtenol, and 2-phenylethanol follow the pattern for *cis*-verbenol. Ipsdienol and ipsenol were never detected in any entrainment (< 2 ng/entrainment, EICP), and cannot play any big role in attack termination or tree-switching.

Oxygenated Compounds from Phloem. Most of the quantified oxygenated compounds have been identified previously in gallery wall phloem (Leufvén and Birgersson, 1987). Among the most prominent compounds are α -terpineol, α -terpinyl acetate, bornyl acetate, terpinen-4-ol, *trans*-thujanol, and one uni-

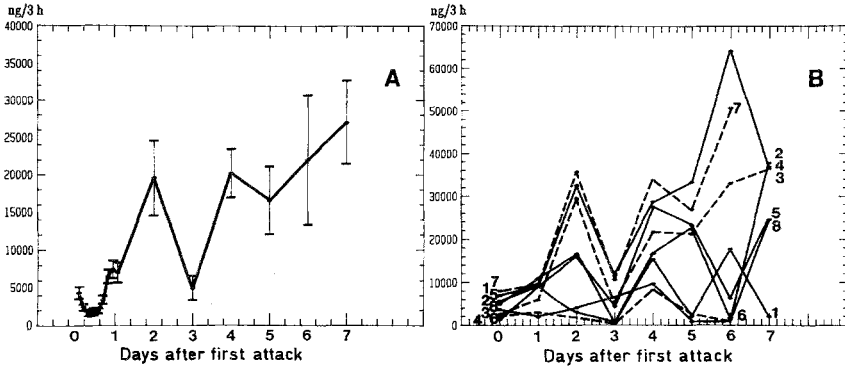


FIG. 4. Amounts ($\mu\text{g}/3 \text{ hr}$) of 2-methyl-3-buten-2-ol collected during the entrainment period. (A) Mean \pm SE. (B) Entrainments from individual entrance holes. -- unmated males, — mated males, respectively, at the end of entrainment period.

identified oxygenated monoterpene (characteristic MS fragments: m/z 53, 81, and 108).³ Other quantified compounds were myrtenal, *trans*-pinocarveol, borneol, verbenone, cryptone, pinocamphone, isopinocamphone, anethole, and camphor. The general patterns for most of these compounds were similar to those for monoterpene hydrocarbons (Figure 6A and C). However, there are discernible differences. For α -terpineol, bornyl acetate, verbenone, and terpinen-4-ol, most of the individuals had a large second increase during days 5–7 (Figure 6B and D). The continuous increase of these compounds seems espe-

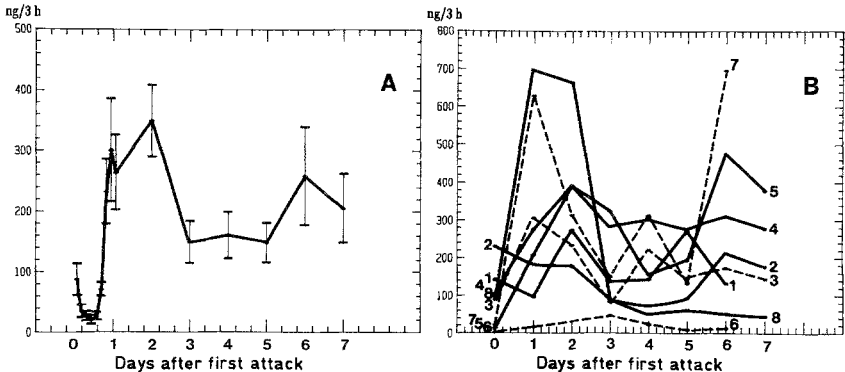


FIG. 5. Amounts ($\text{ng}/3 \text{ hr}$) of *cis*-verbenol collected during the entrainment period. (A) Mean \pm SE. (B) Entrainments from individual entrance holes. -- unmated males, — mated males, respectively, at the end of entrainment period.

³ Now identified as Pinocarvone.

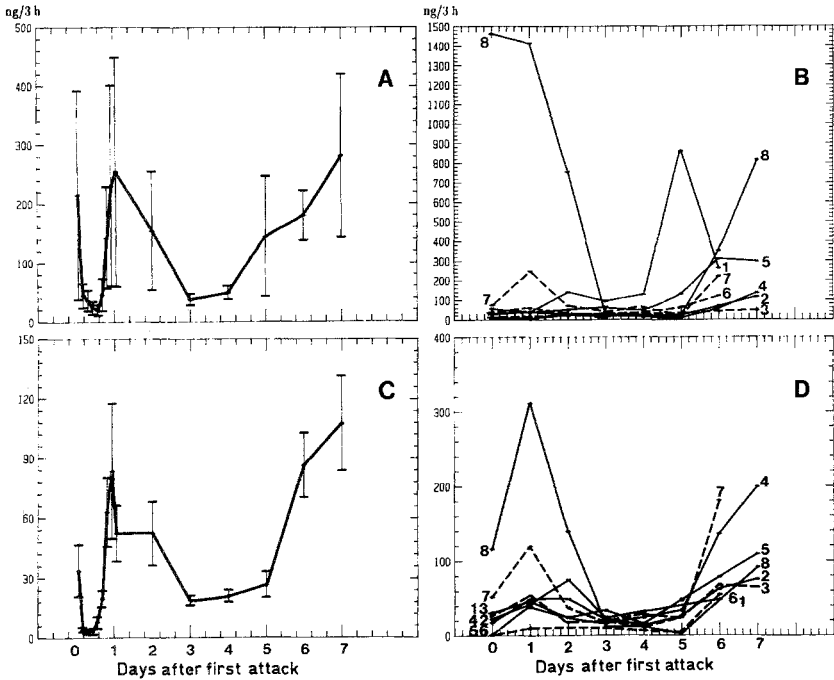


FIG. 6. Amounts (ng/3 hr) of oxygenated monoterpenes collected during the entrainment period. (A) Terpinen-4-ol, mean \pm SE. (B) Amounts of terpinen-4-ol, collected from individual entrance holes. -- unmated males, — mated males, respectively, at the end of entrainment period. (C) Verbenone, mean \pm SE. (D) Amounts of verbenone, collected from individual entrance holes. -- unmated males, — mated males, respectively, at the end of entrainment period.

cially pronounced if their amounts are compared with the emitted amounts of supposed and/or probable monoterpene hydrocarbon precursors (Figure 7).

The release of all oxygenated compounds, except pinocamphone, was much larger from entrance 8 during the first average increase. From this entrance hole, very large amounts of oxygenated compounds were released as early as in the first entrainment only a few hours after the attack was initiated (Figures 6B and D). Only small amounts of these compounds were released from most other entrances during the first few days. However, during the second period of increase at the end of the entrainment period, the released amounts of oxygenated compounds from the gallery wall phloem increased from all entrance holes.

The frass production, visually measured as frass amount in the glass T at

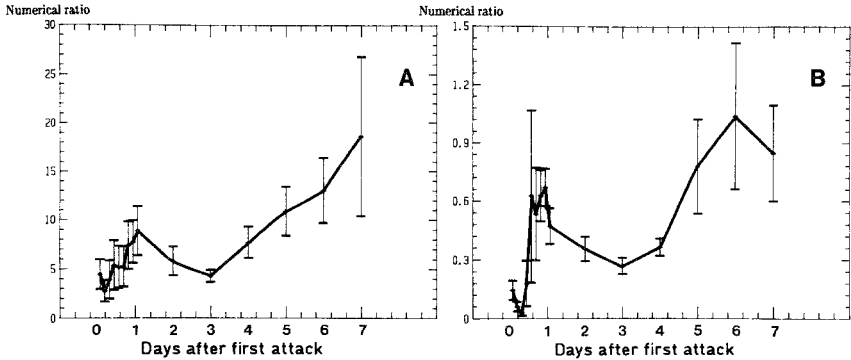


FIG. 7. Average numerical ratio, mean \pm SE, between amounts of collected oxygenated monoterpenes and monoterpene hydrocarbon precursor, during the entrainment period. (A) Terpinen-4-ol (ng/3 hr) : limonene ($\mu\text{g}/3 \text{ hr}$) (B) Verbenone (ng/3 hr) : α -pinene ($\mu\text{g}/3 \text{ hr}$).

the end of the 3-hr entrainment, was lower from the unmated males (Nos. 3 and 7) than from the mated ones during the last days of the entrainment period. When the galleries were cut open after the last entrainment, no attack showed brown-stained gallery wall phloem.

DISCUSSION

When males of *Ips typographus* start to bore into the bark and phloem, they meet the primary resin of the tree. This resin is a part of the tree's defense system (Berryman, 1972), due to both its sticky properties and the content of compounds, such as monoterpene hydrocarbons, which are toxic to the invading bark beetles (cf., Cates and Alexander, 1982). When exposed to monoterpene hydrocarbons, such as α -pinene, by both inhaling and feeding, the males start to produce one of their aggregation pheromone components, (*S*)-*cis*-verbenol (cV), which accumulates in the hindguts (Klimetzek and Francke, 1980). But how soon after the first attack can the beetles release these semiochemicals to attract conspecifics in order to overcome the resistance of their host tree? In addition, males walking on the bark of the tree have been found to release their own essential aggregation pheromone component, 2-methyl-3-buten-2-ol (MB) (J.P. Vité, personal communication).

To quantify the release of semiochemicals, especially the highly volatile MB, from individuals of attacking SBBs, a new method was developed. Vité analyzed the amounts of released MB from walking SBB males in the laboratory, by using a closed-loop stripping apparatus (CLSA) (Lorbeer et al., 1984)

together with active charcoal filters (Bender & Hobein AG, Zürich, Switzerland). This method works very well for trapping MB, but is not so practical for studying naturally attacking bark beetles in the field.

The reason for entraining volatiles from individual entrance holes is to come as close as possible to the source of semiochemicals under natural conditions. We know that there are both quantitative and qualitative variations in volatiles produced by individual bark beetles (Birgersson et al., 1984) as well as in the phloem of gallery walls (Leufvén and Birgersson, 1987). Following the same individuals during the first week of their attack by chemical analysis, we were able to determine such variation within and between individuals.

Average Amounts of Released Components. On average, all the quantified compounds, perhaps with the exception of MB, show similar patterns of variation (Figures 2A, 4A, 5A, 6A and C): two more-or-less pronounced maxima, with an intervening decrease. This general trend closely resembles the findings by Leufvén and Birgersson (1987) for compounds analyzed in phloem tissue around the galleries (cf., Byers, 1981b). There is a decrease in amounts of most volatiles in the phloem surrounding beetles in attack phases 4-5 (for the definition of attack phases, see Birgersson et al., 1984), when males had accepted females.

The amounts of emitted monoterpene hydrocarbons were relatively large in the first entrainment, only a few hours after the males started to bore into the spruce tree. This indicated that the primary resin is mobilized very fast. The amount of monoterpene hydrocarbons is very low in unattacked phloem and rises at least 10-fold when the males bore into it (Leufvén and Birgersson, 1987). For some of the entrances, the first entrainment contained the largest amounts of monoterpene hydrocarbons during the entire entrainment period (Figure 2B). The second increase, which appears during the last two days, likely resulted from the production of new, so-called secondary resin (Christiansen and Horntvedt, 1983). In addition to this newly produced resin, primary resin from the progressive ends of the mother galleries may, to some extent, have contributed to the total amounts of emitted monoterpene hydrocarbons.

The production and release of MB was shown to start very early in the attack (Birgersson et al., 1984) and may even start before the males begin to bore into the phloem (J.P. Vité, personal communication). This is also shown in the first entrainment here, where the released amounts averaged 4300 ng/3 hr. MB has been shown to promote landing (Schlyter et al. 1978b), and this early release can be interpreted as the males are adapted for a fast attack on a selected tree. In general, the release of MB was much larger than expected, according to what has been quantified in SBB male hindguts (Birgersson et al., 1984, 1988). The average of collected amounts during the first entrainment, 4300 ng, can be compared with the amounts found in hindguts by Birgersson et al. (1984) of 100 ng for males boring into the phloem, which rises to 500 ng

when they are excavating their nuptial chambers. The amounts released during 3 hr are at least 10 times larger than the average hindgut content. This implies a very high turnover for the production of MB.

The decrease in released amounts of MB on the third day is significant. One reason for this lower release can be the temperature, which was lower this day than the days before and after (Figure 3). However, this drop in afternoon temperature is considered too small to be the main reason for the lower production and release during the third day after the attack. During the first night, when the temperature fell from 18°C to only a few degrees above freezing, the average amounts of released MB decreased to half of the earlier amounts, which can be directly related to the drop in temperature, as both biological activity and vapor pressure are reduced at lower temperature. The lower production of MB on the third day coincides with the decrease of all quantified compounds, both hydrocarbon and oxygenated monoterpenes, although MB is not assumed to be a metabolite from host tree monoterpenes (Figures 2A, 5A, 6A and C). The most likely reason is that the tree is running out of primary resin, which is the first line of defense. This decrease also coincides with the decrease in male hindgut content, when the males have admitted females (Birgersson et al., 1984).

The significant increase in MB production on the fourth day and the continued large release may be due to the fact that this spruce tree was not taken by the beetles. The production of MB may also be an induced response to the host-tree production of defensive compounds. This interpretation is in agreement with the findings by Birgersson et al. (1988), who found high-MB-producing mated males in resinous spruce trees. Our hypothesis, that male SBBs attacking resistant spruce trees continue to produce aggregation pheromone components, is further supported by the results of Birgersson and Leufvén (1988), who showed that late-attack-phase males surrounded by unstained phloem on the average produce more MB than males surrounded by stained phloem. The brown staining is a sign of fungal establishment in the phloem, indicating that the defense of the tree has been overcome by the bark beetle-fungal attack. This relationship between pheromone production and host tree resistance was also discussed by Raffa and Berryman (1980).

cis-Verbenol is known to be produced from (-)- α -pinene by SBB males (Klimetzek and Francke, 1980). This compound is unique among bark beetles, as it has only been quantitatively determined (Byers, 1989) and shown to elicit attractive response in *Ips* species (Borden, 1982). In addition, cV is possibly an inhibitor of *Dendroctonus brevicomis* (Byers and Wood, 1980). Since Leufvén and Birgersson (1987) did not find cV to be produced in the gallery wall phloem in later attack phases, we assume that all the cV released from the beetle galleries is produced by the beetles themselves, predominantly by the males (Birgersson et al., 1984). Compared with the pattern of its precursor, α -

pinene, the amounts of released cV are low during the first afternoon of the attack (= day 0). It takes one to two days for the beetles to reach their maximum in cV production and release. The efficiency in producing cV from α -pinene, from the first entrainment until the maximum, increases more than five times (Figure 8A). The decrease in the efficiency during the first night and the third day can probably be explained as an effect of the lower temperature (cf., Figure 3). However, the decreasing release at the end of the entrainment period cannot be temperature dependent, as the temperature was increasing during these days. This decreased release of cV in relation to the amount of α -pinene is probably due to microbial activity in the gallery wall phloem, where the beetle-produced cV is converted to Vn (Leufvén et al., 1984). In addition, the microbial activity in the gallery wall phloem, giving increasing amounts of oxygenated compounds, mainly monoterpenes (Birgersson and Leufvén, 1988), influence the attacking bark beetle males to reduce their production of aggregation pheromone components in relation to events in the surrounding phloem.

Several oxygenated monoterpenes in the gallery wall phloem show pronounced quantitative increases during the later attack phases (Leufvén and Birgersson, 1987). Since many of these compounds can be produced by yeasts, isolated from SBB (Leufvén et al., 1988), and the number of these microorganisms increases during the attack (Leufvén and Nehls, 1986), the oxygenated compounds in the phloem were interpreted to be of microbial origin (Leufvén and Birgersson, 1987). In the present study, many of the non-beetle-produced oxygenated compounds were collected in large amounts during the first days of attack. These compounds are unlikely to be produced by microorganisms this early in the attack. Instead, in the beginning of the attack, the only source has

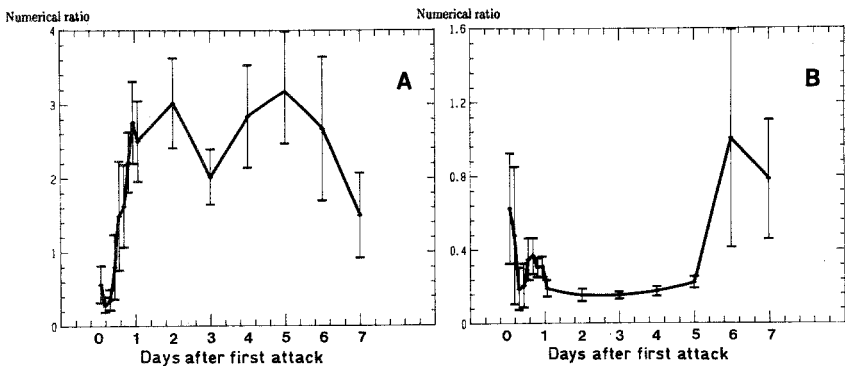


FIG. 8. (A) Bark beetle efficiency in production of *cis*-verbenol from α -pinene during the entrainment period: ratio between *cis*-verbenol (ng/3 hr) and α -pinene (μ g/3 hours), mean \pm SE. (B) Ratio between verbenone and *cis*-verbenol during the entrainment period, mean \pm SE.

to be spontaneous, abiotic oxidation (Leufvén and Birgersson, 1987). A second mechanism for this early production of oxygenated monoterpenes can be through enzymes released from host tree cells, that were injured by the excavating beetles. After the intervening decrease of emitted oxygenated monoterpenes and during the larger second quantitative increase (Figures 6A and C), established microorganisms may have contributed to the production. This is probably also the case, because the oxygenated monoterpenes, compared with the likely precursors, show an increase at the end of the entrainment period (Figure 7).

In the quantitative study by Leufvén and Birgersson (1987), borneol was a prominent oxygenated compound in the gallery wall phloem. In the present study, only trace amounts of borneol have been quantified. However, bornyl acetate, which was only found in trace amounts in the gallery walls, is a prominent compound in the entrainments. From this we infer that borneol in microorganism-inoculated phloem is given off as bornyl acetate.

Variation among Entrance Holes. All the males attacked the same spruce tree at about the same time, but they did not meet the same degree of defense. Most of the beetles met a "medium" defense, as judged by the emission of around 500 μg monoterpene hydrocarbons during the first entrainment (Figure 2B). Two males were faced with very low amounts of monoterpene hydrocarbons, while one male (No. 8) was fighting for three days in a heavy resin flow. After the intervening decrease in emitted monoterpene hydrocarbons, the amounts increased from almost all attacks during the last days, probably due to the production of secondary resin.

The individual patterns for the two aggregation pheromone components, cV and MB (Figures 4B and 5B), differ from each other and from other oxygenated compounds. All males released large amounts of MB (several micrograms per 3 hr) during the first entrainments and did not increase their release the next day (day 1). At the same time, almost all males released low amounts of cV during the first day (day 0) and increased their production and release 10-fold the next day, possibly in order to detoxify the α -pinene they were exposed to in the phloem. If cV is produced as a detoxification product by mixed-function oxidases (Hunt and Smirle, 1988), it could take some time to induce the enzyme system. The male fighting in resin (No. 8) might have been poisoned by the very large amounts of resin, since he did not produce either MB or cV in excessive amounts.

The general decrease of released cV, during days 3–5, cannot be simply temperature dependent, as not all the males decrease their production during these days. However, all males, except No. 8, clearly decrease their MB production on the third day, only to increase it again the next day. It is notable to see that entrances 3 and 7, where the males were still unmated (i.e., attack phase 3) at the end of the entrainment period, are both among the high producers during the last days (Figure 4B).

The very large amounts of monoterpene hydrocarbons present in entrance 8 were probably the main reason for the high release of oxygenated monoterpenes from this entrance (Figure 2B, 6B and D). Terpinen-4-ol predominated from this entrance during the first days; the release from other entrance holes was low (Figure 6B). However, during the second increase, at the end of the entrainment period, all galleries release terpinen-4-ol. Notable is the large amounts of terpinen-4-ol from entrance 8 during the last days (Figure 6B), which can be interpreted as microbial oxidation. The increased production of terpinen-4-ol from its possible precursor, limonene, during the end of the entrainment period, is shown in Figure 7A.

The average release of verbenone (Vn) probably has two maxima; even though No. 8 is predominantly responsible for the first one, Vn is released from most of the entrances. After the intervening decrease, Vn is released from all the galleries during the last days. This increased production of Vn compared with the beetle production of cV yields an increasing ratio between Vn and cV towards the end of the entrainment period (Figure 8B). The reason for this changed ratio is likely the conversion of cV to Vn (Leufvén et al., 1984) by microorganisms established in the gallery wall phloem (Leufvén and Birgersson, 1987).

Released Compounds and Attack Success. Bark beetles attacking a host tree that has not been taken seem to continue their production of aggregation pheromones as long as the tree defends itself by producing secondary resin. It is difficult to determine the attack phase of a male from only the patterns of released compounds from the entrance hole. In Figure 9, the amounts of α -pinene, MB, cV, and Vn trapped outside the galleries of one unmated male (Figure 9A) and three mated ones (Figure 9B-D) are shown. The only difference between the mated and the unmated males is the continually increasing amount of MB released from the unmated males (Figure 9A; cf., Nos. 3 and 7 in Figure 4B). General for all the attacks is the increasing amounts of Vn during the end of the entrainment period. However, the large variations between the studied males might be a result of the fact that the host tree was not taken by the attacking beetles.

Oxygenated compounds are released from the attacks on the first day. For the majority of these compounds, the increase was more pronounced towards the end of the seven-day entrainment period, when they are presumably produced by microorganisms established in the gallery wall phloem. However, this second, and larger, increase appears too late to play a role in the mechanism of tree-switching. It is known from field bioassays that SBB males avoid high concentrations of their own aggregation pheromone components, especially cV (Schlyter et al., 1987b). Probably the large amounts of aggregation pheromones, produced by the male bark beetles, and oxygenated monoterpenes, such as verbenone (Bakke, 1981; Schlyter et al., 1989), as well as possibly α -ter-

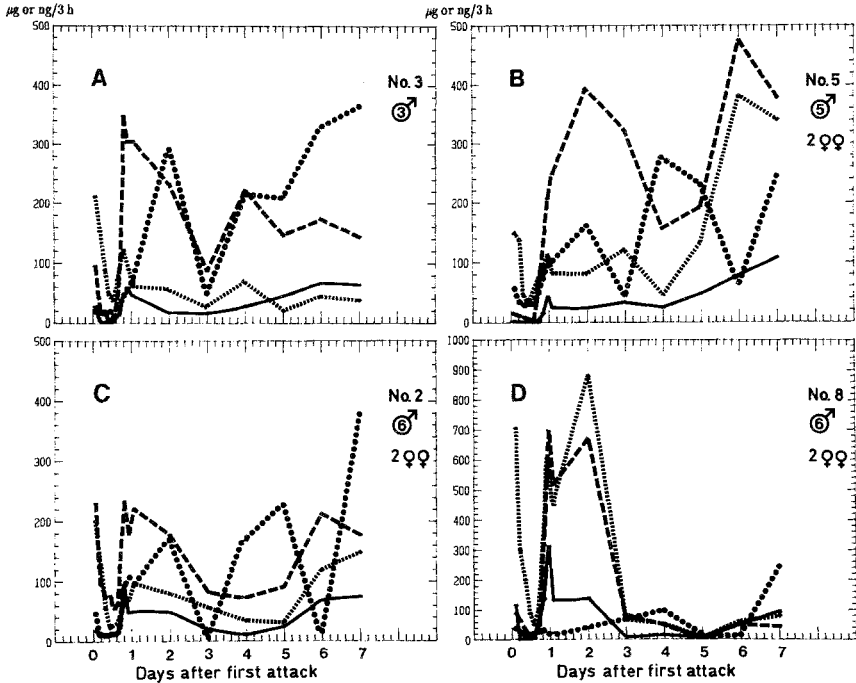


FIG. 9. Amounts of α -pinene (μg) ■■■■■■■■■■, *cis*-verbenol (ng) — — — — —, verbenone (ng) —————, and 2-methyl-3-buten-2-ol (ng/100) ● ● ● ● ● collected from individual entrance holes. (A) No. 3, unmated male; (B–D) Nos. 5, 2, and 8, mated males (for definition of attack phase, see Birgersson et al., 1984). ③ unmated male in nuptial chamber (attack phase 3); ⑤ mated male with females in 0- to 2-cm-long mother galleries (attack phase 5); ⑥ mated male with females in 2- to 4-cm-long mother galleries (attack phase 6).

pineol, terpinen-4-ol, *trans*-pinocarveol, and bornyl acetate, released during the first days of an attack will cause the males to both space out on the bole of the attacked spruce tree and participate in the tree-switching mechanism.

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INTERACTIONS BETWEEN PHEROMONE TRAPS WITH
DIFFERENT STRENGTH LURES FOR THE PINE
BEAUTY MOTH, *Panolis flammea*
(LEPIDOPTERA: NOCTUIDAE)

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Abstract—Catches of male *Panolis flammea* in traps baited with lures containing 25 μg of sex attractant are enhanced when in the proximity of traps baited with 125- μg lures. The degree of enhancement is increased as the intertrap distance is decreased, and when the low-dose trap is upwind of the high-dose one. The patterns of alteration in trap catch suggest that moths initially attracted by one lure may divert into other traps, which may be either upwind of the original trap (“overshooting”), or downwind (“undershooting”). “Overshooting” can result in up to fivefold increases in catch and may provide a useful method for estimating the attractant zone of particular lure/trap combinations.

Key Words—Pine beauty moth, *Panolis flammea*, Lepidoptera, Noctuidae, sex attractant, monitoring trap, chemoorientation, odor plume, trap interference.

INTRODUCTION

Traps, baited with synthetic pheromone lures, are increasingly being used as a survey method for many species of pest Lepidoptera. The relationships among trap catch, population parameters that refer directly to unit area, and subsequent

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insect damage are complex and can usually only be derived empirically, species by species and crop by crop. One of the factors that determines catch is the characteristic of the trap as a sampling device; i.e., the relationship between the local insect population and the probability of an individual insect being caught. This relationship can be split into a number of parts, including the attraction zone of the lure, the attraction and capture efficiencies, the ways in which lure release rates and micrometeorology affect these parameters, and the changing responsiveness of the insects themselves. Remarkably little is known about any of these factors, mainly because it is difficult to account for all of them simultaneously under field conditions.

Several attempts have been made to estimate the dimensions of the attraction zone. Direct observations of caged gypsy moths, *Lymantria dispar*, downwind of an artificial lure have yielded important information on both the distance and time course of effective stimulation of flight (Elkinton et al., 1984). Mark-recapture techniques have also been used (e.g., Elkinton and Cardé, 1980, 1981; Hartstack and Witz, 1981; Schwalbe, 1981; Ramaswamy et al., 1983). However, such experiments are expensive in biological material and cannot reliably estimate the effects of other parameters that affect recapture rates, such as dispersal independent of stimulation by pheromones, and any idiosyncrasies of released insects compared to the undisturbed wild population; the responsiveness of male moths to the female pheromone is notoriously susceptible to handling.

Traps laid out in regular rows or grids have been used to estimate attraction distances, based upon the degree of interference. Estimates for the attraction of codling moth *Cydia pomonella*, obtained in this way, have not tallied with one another (Reidl and Croft, 1974; McNally and Barnes, 1981), which indicates that some unidentified factor must have contributed to relative trap catches. Wall and Perry (1978, 1980) reported that, in lines of two or three traps parallel to the wind direction, baited with (*E, E*)-8,10-dodecadien-1-yl acetate, the upwind trap caught more pea moths *Cydia nigricana* than the average for the line, but the degree of difference was insensitive to intertrap spacings ranging from 25 to 200 m. The bias in favor of the upwind trap disappeared when the lure dose was reduced 100-fold (Wall and Perry, 1981). However, both these lure strengths were equally effective at catching males when deployed simultaneously (Greenway and Wall, 1981), which suggests that the higher dose may have been partially inhibitory at close range, enhancing the probability that moths would move from one trap to another in the multitraps experiments.

In these, and the more complex experiment of van der Kraan and van Deventer (1982), all of the traps were baited with identical doses. In this article we report our attempt to measure perturbations in catch in traps that are in the proximity of other traps baited with lures five times stronger. This arrangement should have two advantages over uniform doses. First, the degree of interfer-

ence should be assymmetric, i.e., the effect of high dose on low dose should be greater than vice versa. Therefore, it should be easier to assign interactions to a particular trap. Second, the lower dose can be chosen to have little attractant power of its own, and hence any increase in catch due to interference by the high dose trap should be easily measured.

The insect chosen was the pine beauty moth *Panolis flammea* (Denis & Schiffermuller) (Lepidoptera: Noctuidae), a pest of lodgepole pine (*Pinus contorta*) plantations. Monitoring traps for this species in Scotland and the north of England are placed in forest rides, which are long, narrow, straight clearings between densely planted areas of pine trees. The male moths normally approach the traps along the axis of the ride (Ellis and Bradshaw, unpublished), probably because the wind tends to be funneled in this direction. Thus traps can be set out in lines that are parallel to the predominant flight directions for most of the time, which simplifies the situation compared to that obtained in an open field and eliminates the need for traps to be realigned with every change of wind direction.

METHODS AND MATERIALS

Two experiments were carried out in consecutive years, in the Elchies block of Craigellachie Forest in Scotland. Large plastic delta traps with removable sticky inserts, 380 cm² in area (Wolfson Unit of Chemical Entomology, Southampton, U.K.), were baited with either 25 μ g or 125 μ g of (*Z*)-9-tetradecenyl acetate, with 10% (*Z*)-11-tetradecenyl acetate added, loaded on to red rubber sleeve stoppers (West Pharmarubber, St. Austell, U.K.). This lure, although not identical to the natural pheromone (Baker et al., 1982), is within the range of blends that are as effective at catching moths in traps (Bradshaw et al., 1983a; Priesner and Schroth, 1983). The doses chosen are well within a regular and positive dose-response relationship for this species, and the 25- μ g lure catches less than 10 moths per night even from economically damaging populations (Bradshaw et al., 1983b). Traps were deployed on posts approximately 1.5 m from soil level, equidistant from the sides of the rides, and with their open ends facing along the ride axis. Each ride selected contained a line of three traps, a single central high-dose (125 μ g) trap flanked by two low-dose (25 μ g) traps (see Figure 1).

In experiment 1, forty such lines of traps were deployed at intertrap spacings of 1, 5, 20, 50, and 100 m. Treatments were allocated to particular rides so as to balance out different compass orientations and the population densities as estimated by pupal surveys in the previous autumn (Holden and Bevan, 1979). The first trap in each line was placed 10 m from the ride end (usually a junction with other rides). Traps were set out between March 15 and 19, before the main

flight period, and sticky inserts were removed for counting and replacement on April 3–4 and April 13–14, and final removal on May 19–20. Catches in the first period exceeded 50 in many traps; since the efficiency of the traps declines above 50 (Bradshaw et al., 1983b; Bradshaw and Ellis, unpublished), the results for this period were excluded. No single trap catch exceeded 50 in the second and third periods.

In experiment 2, three pairs of adjacent east–west rides were selected. Two lines, one of 5 m spacing, and one of 50 m spacing, were assigned at random to each pair of sites. All traps were at least 100 m from any junction between rides. The traps were set out on March 27, and catches were recorded daily until April 19. Most of the catches occurred on six nights when the wind in a seventh parallel ride was recorded as predominantly from the west, during the flight period (1700–1900 hr GMT); on the other nights the wind was predominantly easterly, and temperatures were below the flight threshold. Analysis was performed on the total catches for each trap for these six nights only.

RESULTS

Experiment 1. The total catches from each of the treatments exhibited quite a large range (Table 1), suggesting that the assignment of population densities to sites was imperfect. However, analysis of variance (ANOVA) indicates the overwhelming influence of trap spacing on catch in the low dose traps ($F = 8.98$, $df = 4$, $P < 0.0001$). No significant ($P < 0.10$) effect was found for low-dose trap position (i.e., first or third from the start of the ride). To correct for site-to-site differences in total trap catch, high/(mean low) ratios were calculated for each site (Table 1). These ratios were log-transformed to minimize dependence of the treatment variances on the means. Nested ANOVA on these ratios indicates that the most powerful effect is from spacing ($F = 9.13$, $df = 4$, $P < 0.0001$). The value of the ratio increases with distance up to 50 m, while at 100 m it declines slightly (Table 1). This can be interpreted in terms of an increasing proportion of the moths that are initially attracted by the high-dose trap being caught by the flanking low-dose traps as the distance between traps is decreased from 50 m.

Experiment 2. The first experiment gave no indication as to the mechanism whereby the low-dose trap catch was enhanced by proximity to a high-dose trap. Catches for nights with westerly winds in the second experiment indicate that upwind low-dose traps caught considerably more moths than downwind low-dose traps (Table 2). This effect was more pronounced for the 5-m spacing (as expected from the results of the previous experiment), and ANOVA on the low-dose catches indicates significant effects for both spacing and orientation to the

TABLE 1. MEAN CATCHES OF MALE *Panolis flammea* IN LINES OF THREE TRAPS (EXPERIMENT 1)^a

Spacing (m)	N	Mean trap catch				Mean log (high catch/mean low catch)
		Low 1	High	Low 2	Total	
1	8	7.3	15.1	6.4	28.7	0.322a
5	8	3.4	11.9	2.8	18.1	0.569ab
20	8	3.2	23.8	3.9	30.8	0.861c
50	8	3.6	23.5	1.7	28.8	1.027c
100	8	2.3	16.0	2.4	20.6	0.857bc

^aHigh/mean low ratios were calculated for individual sites and catching periods, and converted to \log_{10} before calculating means. Ratios followed by the same letter are not significantly different by Duncan's new multiple-range test at $P = 0.05$.

TABLE 2. MEAN CATCHES OF MALE *P. flammea* IN GROUPS OF THREE TRAPS AT 5 m AND 50 m SPACINGS (3 REPLICATES EACH, SIX NIGHTS WITH WESTERLY WINDS)^a

Spacing (m)	Mean trap catch				Mean log (high catch/mean low catch)
	Low (upwind)	High	Low (downwind)	Total	
5	14.7a	24.3	4.7b	43.7	0.401
50	3.0bc	29.7	1.3c	34.0	1.238

^aLow-dose trap catches followed by the same letters are not significantly different by Duncan's new multiple-range test at $P = 0.05$.

wind (Table 2). Catches in both upwind and downwind low-dose traps at 5 m were enhanced compared with those at 50 m. The mean high/(mean low) ratios were numerically similar to those obtained for the same spacings in Experiment 1, suggesting that the same mechanism for enhancement of the low-dose traps was in operation for the two years.

DISCUSSION

Interpretation of these complex interaction effects is simplified if it is assumed that, in experiment 2, the low-dose traps that are 50 m upwind of a high-dose trap are the least affected by the proximity of other traps. That this

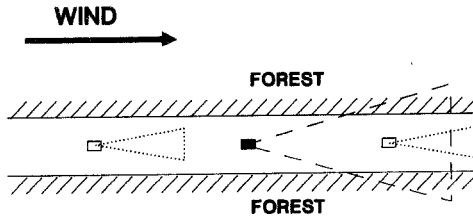


FIG. 1. Diagram showing the relative positions of traps at 50-m spacing in experiment 2 and their inferred attraction zones, with wind blowing from left to right. The trap containing the high-dose lure ($125 \mu\text{g}$) is shown as a solid square, those containing low doses ($25 \mu\text{g}$) by open squares. The borders of the ride are shown shaded.

is likely is illustrated in Figure 1. The catch in the other low dose traps can be interpreted as follows:

Upwind Trap at 5 m. The catch is considerably enhanced by its proximity to the high-dose trap, probably because many moths, originally attracted towards the high-dose trap, overshoot and proceed the short distance upwind to be caught by the low-dose trap.

Downwind Trap at 50 m. The small (just nonsignificant) depletion of catch compared to the upwind trap at 50 m can be explained by the same mechanism; some moths attracted towards the downwind low-dose trap overshoot and proceed upwind towards the high dose one.

Downwind Trap at 5 m. There is apparently a balance between overshooting (depleting catch) and additional recruitment of moths due to the proximity of the high-dose lure (i.e., undershooting).

This interpretation is at least qualitatively supported by observations of the behavior of *P. flammea* (Bradshaw and Ellis, unpublished), which is similar to that of many other noctuids (e.g., *Spodoptera littoralis*; Murlis and Bettany, 1977). Males accidentally flying past a pheromone source during their upwind approach frequently cast back downwind to relocate the pheromone plume. In the experiments described here, however, after overshooting, the male may find itself within the interference zone of another trap upwind, provided that trap is sufficiently close, and will then tend to proceed upwind, increasing the catch in the upwind trap and decreasing the catch in the trap that the moth originally approached. There may also be a contribution from "plume-switching" away from the vicinity of the traps.

The data from experiment 1 (Table 1) are consistent with this interpretation. The high/mean low ratio is a composite function of effects on all three traps, but is predominantly due to the probability of moths that overshoot the high-dose trap being attracted on to the upwind low-dose trap. At some intertrap

distance greater than 5 m, but almost certainly less than 50 m, the probability of this occurring becomes negligible. At this distance there is still a significant probability that moths overshooting the downwind trap will still be attracted to the high-dose trap, but at some greater distance all three traps should become independent. This will cause the high/(mean low) ratio to fall again, and this is possibly observed at the 100-m spacings.

This mechanism is similar to that proposed by Lundberg and Lofstedt (1987) to model competition between "calling" females; they also demonstrated, in wind-tunnel experiments, that a low-dose lure 1 m upwind of a high-dose lure could successfully attract males of *Yponomeuta cagnagellus*. Wall and Perry (1978, 1987) and Perry and Wall (1984a,b) proposed similar mechanisms to account for enhanced catches of *C. nigricana* in upwind traps containing identical high doses to downwind traps. We have here demonstrated the same effect for lure doses that fall within an increasing dose-response relationship and may therefore more naturally mimic the calling female. The asymmetric arrangement of lure doses has made the effect more obvious and provides a useful method for determining the interference distance of a particular lure. For *P. flammea*, this method gives an estimate of between 5 and 50 m, and probably in the lower part of this range, for the effective interference distance of a 25- μ g lure under the conditions of the experiments described. Since this distance is likely to be affected both by wind strength and local topography (Perry and Wall, 1984a), it may vary with location, but should be useful in interpreting catches in standard (25 or 5 μ g baited) monitoring traps for this species.

The relationship between the interference distance (i.e. attraction from just upwind of a higher-dose lure) and the attraction distance (i.e. attraction in the absence of other synthetic lures) remains to be established. They need not be identical; the behavior of a moth when it overshoots a high-dose lure may differ from its appetitive flight behavior (Elkinton and Cardé, 1983; Sabelis and Schippers, 1984) or flight which has not been stimulated so recently by high concentrations of pheromone.

These experiments also indicate that trap spacing will have a profound effect on the dose-trap-catch relationships obtained for this (Bradshaw et al., 1983b) and presumably many other species, when traps containing different doses have been sufficiently close for interference to occur (e.g., Chisholm et al., 1979; Flint et al., 1978; Heath and Landolt, 1987; Kraemer et al., 1979, 1983; McNally and Barnes, 1980; Olaifa et al., 1984; Roelofs et al., 1977, 1982; Turgeon et al., 1983; Vick et al., 1979; Yonce et al., 1976). In particular, the slopes of the lines obtained may need reevaluating, in view of the fivefold difference in catch ratio for the same pair of doses, as reported here. There may also be effects on trials designed to evaluate optimum blends of pheromone components where the traps are placed within their mutual interference distances.

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SEX PHEROMONE COMPONENTS OF THE
BLUEBERRY LEAFTIER MOTH *Croesia curvalana*
KEARFOTT (LEPIDOPTERA: TORTRICIDAE)

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Abstract—The GC-MS and GC-EAD analyses of sex-gland components and sex-gland-released volatiles have identified (*E*)- and (*Z*)-11-tetradecenal (90:10) and (*E*)- and (*Z*)-11-tetradecenyl acetate (85:15) as components of the sex pheromone chemical communication system of *Croesia curvalana* (Kearfott). The aldehyde-to-acetate ratio of 9:1 was found in the gland-released components. This blend at a 0.1–1 µg/day release rate from PVC lures was shown to be as effective as virgin females in the trapping of male *C. curvalana*.

Key Words—Lepidoptera, Tortricidae, *Croesia curvalana*, blueberry leaftier moth, sex pheromone components, (*E*)-11-tetradecenal, (*Z*)-11-tetradecenal, (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate.

INTRODUCTION

Croesia curvalana is a destructive pest of low-bush blueberry crops in north-eastern North America (Morris, 1981). Eggs hatch in early spring (Ponder and Seabrook, 1988), and newly emerged larvae burrow into the protective flower buds making insecticide control difficult. Identification of the pheromone system of the adult moth may have potential for management of this species.

Aldehydes and acetates have been found to be attractants and primary pheromone components in the sex pheromone systems of the subfamily Tortricinae,

which includes *Croesia* and *Choristoneura* (Booij and Voerman, 1985; Ando et al., 1981; Grant et al., 1981; Inscoe, 1982). However, the chemical identification of sex pheromone components in the *Croesia* sp. has not been reported to date.

In the course of conducting trapping studies with *Choristoneura fumiferana* (spruce budworm), it was observed that *C. curvalana* males were attracted to traps baited with virgin *C. fumiferana* females, which release a blend of (*E*)-11-tetradecenal, (*Z*)-11-tetradecenal, tetradecanal, and (*E*)- and-(*Z*)-11-tetradecenyl acetate (95:5) in a ratio of 95:5:2:0.2, respectively (Silk et al., 1980). This indicated the possibility that the pheromone systems of these two species contained similar pheromone components.

Identification of the major and some minor pheromone components has been accomplished by GC-EAD and GC-MS analysis of the female sex gland constituents as well as gland-released volatiles. The structural assignments are supported by field-trapping studies.

METHODS AND MATERIALS

Insects. *C. curvalana* pupae were collected from the field, separated by sex, and incubated under a 17:7 light-dark cycle at $21 \pm 2^\circ\text{C}$, and 70% relative humidity until emergence. Adult moths were maintained under similar environmental conditions.

C. fumiferana were obtained as second-instar larvae from Great Lakes Forestry Research Laboratory, Saulte Ste. Marie, Ontario, and were maintained on the above environmental regime throughout the larval, pupal, and adult stages.

Choristoneura pinus pinus (Jack pine budworm) adults were field collected in central New Brunswick in late July.

Chemicals. Compounds employed as analogs and reference standards were synthesized using established procedures (Loneragan, 1986) and include the following: (*E*)-12-tetradecenyl acetate (*E*12-14:Ac), (*E*)-11-tetradecenyl acetate (*E*11-14:Ac), (*Z*)-11-tetradecenyl acetate (*Z*11-14:Ac), (*E*)-9-tetradecenyl acetate (*E*14:Ac), (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac), (*E*)-5-tetradecenyl acetate (*E*5-14:Ac), (*Z*)-5-tetradecenyl acetate (*Z*5-14:Ac), tetradecanyl acetate (14:Ac), 13-tetradecenal (Δ 13-14:Ald), (*E*)-12-tetradecenal (*E*12-14:Ald), (*E*)-11-tetradecenal (*E*11-14:Ald), (*Z*)-11-tetradecenal (*Z*11-14:Ald), (*E*)-9-tetradecenal (*E*9-14:Ald), (*Z*)-9-tetradecenal (*Z*9-14:Ald), (*E*)-7-tetradecenal (*E*7-14:Alc), (*Z*)-7-tetradecenal (*Z*7-14:Ald), tetradecanal (14:Ald), and (*E*)-11-tetradecenol (*E*11-14:OH). Sample purity was greater than 95% with geometrical isomer purity greater than 99% (Houx et al., 1974), as determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques.

Compounds used for the field studies were formulated in polyvinyl chloride (PVC) lures at 0.03% active ingredient (Fitzgerald, 1973; Sanders, 1981). The 1 × 0.4-cm PVC lures were aged under field conditions for two weeks, placed in Pherocon 1C traps (Zoecon Corp., Palo Alto., California) and set out in the field experiments.

Effluvia Collection. A slightly modified version of Baker's single-insect-effluvia collection technique was employed for the collection of sex-gland-released volatiles (Baker et al., 1981). The abdominal-gland portion of the female moth was ligated at the second segment, severed, and placed in a constricted, open-ended tube (Eppendorf disposable pipet tube). The gland was extruded by applying gentle pressure with a small glass rod. Four of these gland preparations were placed in the glass collection device, and nitrogen gas (HP grade passed through 4 Å molecular sieve and activated charcoal) was passed over the extruded glands at a flow of approximately 1 ml/min for a 2-hr period during the first 2 hr of the scotophase (period of peak female attractiveness to males; Ponder and Seabrook, 1988). The released volatiles were adsorbed onto two small, precleaned, glass wool filters connected in series. At the end of the collection period, the walls of the collector and the glass wool filters were washed with 2 ml of purified hexane. The collected material was stored in hexane at -20°C until analysis.

Gland Extraction. Fresh glands were severed, homogenized, stored in hexane at 4°C for 48 hr, filtered, and the hexane-soluble material stored at -20°C until analysis.

Instrumentation. GC analyses were performed on a Varian 6000 instrument equipped with a flame ionization detector (FID) and a split/splitless injector. Helium was used as the carrier gas. SPB-5 (30 m, 0.32 mm ID, 0.2 µm film), SP-2340 (60 m, 0.24 mm ID, 0.2 µm film), and SP-2330 (30 m, 0.32 mm ID, 0.2 µm film) capillary columns were employed.

GC-MS analysis was performed on a Finnigan 4021 EI-CI-INCOS system. Helium was used as the carrier gas. An SP-2330 column was utilized; temperature program was 50°C to 200°C at 15°C/min and 0.85 kg/cm pressure. Injections were made in the splitless mode. The EI mass spectra were generated at 70 eV.

GC-EAD Analysis. The sex pheromone gland extracts and volatiles were analyzed by the gas chromatography-electroantennogram detector (GC-EAD) technique (Struble and Arn, 1984), a procedure combining gas chromatography with FID detection and electroantennogram detection (EAD). A modified method (Kuenen, unpublished) was employed in which the effluent from the GC capillary column was split equally and delivered simultaneously to the FID and antennal detectors. The antenna was positioned between the tips of two glass capillaries containing saline solution (Roelofs, 1984) and chloridized silver wire electrodes that were connected to a 10× DC amplifier. The GC effluent

exited into an air stream (flow approx. 50 ml/min, approx. 100% relative humidity) passing over the antennal preparation. The antennal and FID responses were recorded in synchrony on a Honeywell dual-channel chart recorder.

Responses to gland extracts and gland volatiles were recorded from *C. curvalana* antennae to determine electrophysiologically active components. In addition, *C. fumiferana* and *C. pinus pinus* antennal detectors were used to estimate isomer ratios and to corroborate structural assignments of the two major EAD active components.

Field Studies. Tests were conducted at Blackville, New Brunswick, using Pherocon 1C traps baited with lures releasing test compounds. These compounds singly or in combinations were incorporated into PVC lures at concentrations designed to produce release rates of approximately 0.1 to 1 $\mu\text{g}/\text{day}$ (Sanders, 1981).

Traps baited with virgins had two 0- to 24-hr-old females placed in a 5 \times 3-cm screened cage made from clear plastic pill vials. All lures were placed on bottoms of traps. The light-dark rearing cycle of females used as baits was synchronous with field conditions. Female baits were replaced every 48 hr, and PVC lures were renewed after two weeks in all tests. Traps were spaced 30 m apart at a height of approximately 40 cm such that trap bottoms were at the blueberry canopy level. Initial positioning of traps was randomized. Trap captures were counted every 24 or 48 hr, depending on the experiment, and the traps were moved forward by one position at this time. The trap bottoms were changed every five days or when more than 20 moths were present.

Test 1. In 1981, E11-14:Ald, Z11-14:Ald, E- and Z11-14:Ac (95:5) and 14:Ald were tested singly and in combinations in arrays that included traps baited with two virgin *C. curvalana* and two virgin *C. fumiferana* females. This experiment was replicated in four fields separated by at least 100 m and monitored throughout a 26-day flight period. The data were analyzed using the Tukey multiple comparison test (Zar, 1984).

Test 2. In 1982, attractancy studies were carried out to determine the optimum E:Z ratio of the Δ 11-14:Alds and to ascertain the effect of the addition of varying amounts of 11-14:Ac on these blend ratios. Four blends of E and Z11-14:Ald (95:5, 90:10, 85:15, and 80:20) were tested alone and with the addition of 0.2 and 2% E- and Z11-14:Ac (95:5). The trapping test was run, at one site only, for 22 days.

Test 3. In 1984, field studies were conducted in which the two most attractive E:Z aldehyde ratio blends of the 1982 study were repeated but with the addition of a wider concentration range of the 11-14:Ac components. This test was run for 14 days during the height of the flight season. The data from tests 2 and 3 were subjected to analysis using nonparametric two-way ANOVA (Zar, 1984).

RESULTS AND DISCUSSION

GC-EAD Analysis. GC-EAD analysis of a portion of the hexane gland extract as well as the hexane-soluble gland-released volatiles showed the presence of a number of electrophysiologically active components. The two most active compounds displayed retention times identical to those of *E*11-14:Ald and *E*11-14:Ac (Table 1). Since the SP-2340 column provides separation of *E* and *Z* isomers by at least 0.3 min under the conditions employed, the major components are assigned the *E* configuration. However, additional antennal responses were elicited by components with retention times corresponding to *Z*11-14:Ald and *Z*11-14:Ac. Employing *C. curvalana* antennal detectors, the comparison of the EAD response produced by 11-14:Ald in the gland extract with the responses to synthetic standards of known ratios suggested that the *E*:*Z* ratio of the active aldehyde components was approximately 85:15. The ratio of Δ 11-14:Alds to Δ 11-14:Ac in the gland was estimated to be 1:1 based on the comparison of gland-extract-induced antennal responses with GC-EAD responses to standards of known concentrations and ratios. Extrapolation of the EAD dose-response data leads to the estimate of 60-90 pg of *E*- and *Z*11-14:Ald (85:15) and 60-120 pg of Δ 11-14:Ac present in each female sex gland (3-4 days of age) (Table 2).

In addition to the detection of the above unsaturated aldehydes and acetates, the GC-EAD study indicated the presence of an electrophysiologically active gland component at a retention time corresponding to the saturated aldehyde, tetradecanal (Table 1). The amplitude of the antennal response generated by this compound, when compared with that of synthetic 14:Ald, indicated its concentration in the gland to be roughly equivalent to that of the unsaturated aldehyde, *E*11-14:Ald (60-90 pg; Table 2).

Similarly, GC-EAD analysis of the effluvia also detected the presence of Δ 11-14:Alds and Δ 11-14:Ac. However, an aldehydes-to-acetates ratio of 9:1 was found in the effluvia as opposed to the 1:1 ratio in the gland. Also the *E*:*Z* ratio of the Δ 11-14:Alds was estimated to be 90:10, contrasting with the 85:15 ratio in the gland. The release rate of the major effluvial compound *E*11-14:Ald was estimated to be approximately 30 pg/hr/female (Table 2). GC-EAD confirmation of the presence of 14:Ald in the effluvia was inconclusive. A response was generated at the correct retention time in one effluvial sample but not in two others.

Investigation of gland effluvial components employing *C. fumiferana* and *C. pinus pinus* antennal detectors supported the structural assignments. The primary pheromone components of *C. fumiferana* are *E*- and *Z*11-14:Ald (95:5) (Silk et al., 1980; Sanders and Weatherston, 1976), while those of *C. pinus pinus* are *E*- and *Z*11-14:Ac (85:15) (Silk et al., 1985). The presence of Δ 11-14:Ac in the gland extract and effluvia was detected by *C. pinus pinus* antennae

TABLE 1. GC RETENTION TIMES OF SEX-GLAND-DERIVED MATERIAL MEASURED WITH FID AND EAD

GC column/ standards	Standards Retention (min) ^a	Gland extract retention (isomer ratio) ^b [Ald to Ac ratio] ^c	Effluvia retention (isomer ratio) [Ald to Ac ratio]	Antennal detector (sensitivity for)
SP-2340 ^d				
14:Ald	16.70	16.70 [1]		<i>C. curvalana</i> (Ald and Ac)
E11-14:Ald	17.30	17.30 (85) [1]	17.30 (90) [9]	
Z11-14:Ald	17.65	17.65 (15)	17.65 (10)	
E11-14:Ac	18.01	18.01 (85) [1]	18.01 (85) [1]	<i>C. pinus pinus</i> (Ac)
Z11-14:Ac	18.32	18.32 (15)	18.32 (15)	
SP-2330 ^e				
14:Ald	6.10	6.10 [1]		<i>C. curvalana</i> (Ald and Ac)
E11-14:Ald	6.37	6.37 [1]	6.37 [9]	
Z11-14:Ald				
E11-14:Ac	6.95	6.95 [1]	6.95 [1]	
Z11-14:Ac				
SPB-5 ^f				
14:Ald	15.53			
E11-14:Ald	15.44	15.44 [1]	15.44 [9]	<i>C. fumiferana</i> (Ald)
Z11-14:Ald	15.55			
E11-14:Ac	18.50	18.50 [1]	18.50 [1]	<i>C. pinus pinus</i> (Ac)
Z11-14:Ac	18.65			

^a Retention times of standards were first determined by flame ionization detection.

^b Ratio of double bond geometrical isomers (*E*:*Z*) was estimated by comparison of EAD responses of biological samples with synthetic samples of known *E*:*Z* ratios.

^c Component ratios were estimated by comparison of EAD responses of biological samples with synthetic samples of known blends of aldehydes and acetates and by GC-MS analysis.

^d Temperature program: 50°C (0.5 min) to 200°C at 10°/min; head pressure 1.5 kg/cm².

^e Temperature program: 50°C (0.5 min) to 170°C at 25°/min; head pressure 1 kg/cm².

^f Temperature program: 50°C (0.5 min) to 200°C at 10/min; head pressure 1.5 kg/cm².

(Table 1). The determination of the 85:15 *E*:*Z* ratio of the Δ 11-14:Ac found both in the gland and the effluvia was primarily based on the responses of the *C. pinus pinus* antennae. The *C. curvalana* antennae were not sufficiently responsive to the acetate component to provide quantitative discrimination of the *E* and *Z* acetate isomers.

GC-MS Analysis. Partial mass spectrums of the major active components

TABLE 2. EAD RESPONSES^a OF *C. curvalana* TO SYNTHETIC STANDARDS AND SEX GLAND COMPONENTS^b

	Stimulus concentration		
	1 ng	0.1 ng	0.01 ng
Acetates			
<i>E</i> 12-14: Ac	0.2 ± 0.1	0	
<i>E</i> 11-14: Ac	1.8 ± 0.3	1.0 ± 0.3	
<i>Z</i> 11-14: Ac	0.4 ± 0.1	0.2 ± 0.1	
<i>E</i> 9-14: Ac	0.8 ± 0.2	0.2 ± 0.1	
<i>Z</i> 9-14: Ac	0.6 ± 0.2	0.2 ± 0.1	
<i>E</i> 5-14: Ac	0.3 ± 0.1	0.1 ± 0.1	
<i>Z</i> 5-14: Ac	0.4 ± 0.1	0	
14: Ac	0.5 ± 0.2	0.1 ± 0.1	
0.3 FE effluvia (1 pg)		0.1 ± 0.05 <i>N</i> = 5	
0.3 FE gland extract (20-40 pg)		0.5 ± 0.1 <i>N</i> = 5	
Aldehydes			
13-14: Ald	0.3 ± 0.1	0.1 ± 0.1	0
<i>E</i> 12-14: Ald	1.8 ± 0.3	1.2 ± 0.2	0.3 ± 2
<i>E</i> 11-14: Ald	6.0 ± 0.3	4.3 ± 0.1	2.1 ± 0.5 <i>N</i> = 5
<i>Z</i> 11-14: Ald	2.0 ± 0.5	1.8 ± 0.2	0.6 ± 0.2
<i>E</i> 9-14: Ald	1.0 ± 0.2	0.6 ± 0.1	0.3 ± 0.2
<i>Z</i> 9-14: Ald	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.05
<i>E</i> 7-14: Ald	0.5 ± 0.2	0.2 ± 0.1	0.1 ± 0.05
<i>Z</i> 7-14: Ald	0.5 ± 0.05		
14: Ald	1.6 ± 0.3	1.2 ± 0.3	0.3 ± 0.1
<i>E</i> 11-14: OH			0.6 ± 0.2
0.3 FE effluvia (10 pg)			2.25 ± 0.4 <i>N</i> = 5
0.3 FE gland extract (20-30 pg)			3.05 ± 0.5 <i>N</i> = 5

^a Mean EAD Responses mV ± SD; *N* = 3 except where noted.

^b Analysis performed on a SP-2330 capillary column; temperature program 50° (0.5 min) to 170°C at 15°/min; head pressure 1 kg/cm².

were obtained (Table 3). In the case of the gland extract, a full mass spectrum of 14: Ald was produced (*M*⁺212). Although lacking definitive molecular ions, characteristic mass ion fragments were found for *E*11-14: Ald and *E*11-14: Ac at 192 (*M* - 18) and 194 (*M* - 60), respectively. Analysis of the effluvia sample provided a partial spectrum, displaying a low mass fragmentation pattern similar to that of *E*11-14: Ald, at the correct retention time.

Test 1. Mean daily trap capture of male *C. curvalana* in traps baited with two virgin *C. curvalana* females, two virgin *C. fumiferana* females, and PVC lures releasing *E*11-14: Ald, *Z*11-14: Ald, 14: Ald, and *E* and *Z*11-14: Ac (95:5) are given in Table 4. The results of this study indicate that the four-component blend of *E*11-14: Ald, *Z*11-14: Ald, and *E*- and *Z*11-14: Ac (95:5)

TABLE 3. MASS FRAGMENTS OF *C. curvalana* DERIVED MATERIAL^a

	Gland extract	Effluvia
14:Ald	M ⁺ 212 (M-18) 194 base peak 57, 68, 82 96, 110, 124, 137, 168	
E11-14:Ald	(M-18) 192 base peak 55, 69, 81 98, 111, 121, 135, 149 163	Base peak 55; 69, 81, 98, 111.
E11-14:Ac	(M-60) 194 55, (CH ₃ COOH ₂ ⁺)61 base peak 68, 82, 96, 110, 124, 138, 152, 166	

^aDetermined by GC-MS Finnigan 4021 system. EI-induced ionization analyzed in full-scan mode.

TABLE 4. MEAN DAILY CATCH OF MALE *Croesia curvalana* IN FOUR FIELDS^a BY PHEROCON 1C TRAPS BAITED WITH VIRGINS AND WITH PVC LURES RELEASING SYNTHETIC TEST COMPOUNDS^b

Lure	Males/trap/day ^c
2 <i>C. fumiferana</i> virgins	33.7a
E, Z11-14:Ald + 14:Ald + Δ11-14:Ac	20.3b
E11-14:Ald + Z11-14:Ald + Δ11-14:Ac	19.5b, c
2 <i>C. curvalana</i> virgins	17.4c, d
E11-14:Ald + Δ11-14:Ac	16.0d
14:Ald	14.4e
E11-14:Ald + Z11-14:Ald	12.9e
E11-14:Ald + Z11-14:Ald + 14:Ald	11.1f
E11-14:Ald + 14:Ald + Δ11-14:Ac	10.7f
14:Ald + 11-14:Acs	10.3 f
Δ11-14:Acs	7.8 g
E11-14:Ald + 14:Ald	6.4 h
E11-14:Ald	6.0 h, i
Z11-14:Ald	5.4 i, j
Z11-14:Ald + 14:Ald + Δ11-14:Acs	5.1 j, k
Z11-14:Ald + 14:Ald	4.4 j, k
Z11-14:Ald + Δ11-14:Acs	4.6 k

^aOne of each trap type was replicated in four fields for 26 days of the flight season.

^bRelease rates and ratios of combinations based on that of *C. fumiferana* pheromone blend: E11-14:Ald, 0.1-1 μg/day; Z11-14:Ald, 5-50 ng/day; 14:Ald, 2-20 ng/day; Δ11-14:Acs (E:Z, 95:5) 0.2-2 ng/day.

^cTrap catches followed by the same letter are not significantly different (Tukey multiple comparisons test. α = 0.05).

TABLE 5. 1982 TRAP CAPTURE OF MALE *Croesia curvalana* BY PHEROCON 1C TRAPS BAITED WITH PVC LURES RELEASING SYNTHETIC TEST COMPOUNDS^a

Lure		\bar{X} male capture per 48 hr	Standard error
Ratio of <i>E</i> and Z11-14:Ald	Percent Δ 11-14:Acs ^b		
95:5	0	17.5	6.0
90:10	0	32.3	4.2
85:15	0	7.5	2.3
80:20	0	3.1	0.8
95:5	0.2	57.4	18.4
90:10	0.2	100.0	34.5
85:15	0.2	47.9	15.2
80:20	0.2	4.9	1.7
95:5	2.0	157.3	35.1
90:10	2.0	183.6	32.2
85:15	2.0	129.0	34.4
80:20	2.0	14.1	4.5
Unbaited trap		3.7	1.3

^aPVC release rate = 0.1-1.0 μ g/day of 11-14:Ald.

^b Δ 11-14:Acs (*E*:Z, 95:5).

was equivalent in trapping activity to two virgin *C. curvalana* females. Caution should be exercised when making direct comparisons between trap capture by live virgin females and PVC lures because of the effect of environmental factors (Sanders and Lucuik, 1972; Sanders, 1981). Later studies have indicated that female *C. curvalana* virgins mate in the laboratory at 1-3 days of age (Ponder and Seabrook, 1988). Virgin *C. curvalana* females of more advanced age might have resulted in increased trap captures. It is interesting to note the apparent ability of 14:Ald to trap male *C. curvalana* when presented alone, although it contributed nothing to trap capture when present in other component combinations, with the possible exception of its addition to 11-14:Ac.

Test 2. The 1982 study (Table 5) demonstrated that the blend *E*11-14:Ald, Z11-14:Ald, Δ 11-14:Acs (90:10:2) was the most effective trapping combination at the 0.1- to 1- μ g/day release rate. As the concentration of the Δ 11-14:Acs in the blend was increased, the activity differential between the *E* and Z11-14:Ald ratios decreased. This indicated the upper limit of the activity threshold for Δ 11-14:Ac may not have been reached.

Test 3. In the 1984 study (Table 6) the two most effective *E*:Z blends of Δ 11-14:Ald from 1982 were again tested alone and with quantities of Δ 11-14:Ac ranging from 0% to 40%. The highest moth catches were obtained with the 2% acetate blend. The results of the 1982 and 1984 tests clearly show that,

TABLE 6. 1984 TRAP CAPTURE OF MALE *Croesia curvalana* BY PHEROCON 1C TRAPS BAITED WITH PVC LURES RELEASING SYNTHETIC TEST COMPOUNDS^a

Lure			
Ratio of <i>E</i> and Z11-14:Ald	Percent Δ 11- 14:Ac ^b	\bar{X} male capture per day	Standard error
95:5	0	26.7	9.4
90:10	0	59.3	18.0
95:5	0.2	35.7	7.5
90:10	0.2	49.2	15.5
95:5	2.0	49.2	22.3
90:10	2.0	70.4	18.0
95:5	10.0	28.2	9.4
90:10	10.0	53.1	13.4
95:5	20.0	14.9	5.1
90:10	20.0	57.7	20.3
95:5	40.0	14.8	6.3
90:10	40.0	28.1	9.0
Unbaited trap 2 virgin <i>C.</i> <i>curvalana</i> females		2.1 45.2	1.5 7.4

^aPVC release rate = 0.1-1.0 μ g/day of 11-14:Ald.

^b Δ 11-14:Ac (*E*:*Z*, 95:5).

within any given acetate level, the *E*:*Z* aldehyde ratio of 90:10 was the more attractive blend and that the optimum loading quantity of Δ 11-14:Ac is between 2% and 10% of the total blend (nonparametric two-way ANOVA; *P* (0.001).

CONCLUSION

The sex pheromone components (*E*)- and (*Z*)-11-tetradecenal (90:10) and (*E*)- and (*Z*)-11-tetradecenyl acetate (85:15), have been identified for *Croesia curvalana*. These compounds have been found in the sex-gland-released volatiles and were shown to be electrophysiologically active. In addition, they were formulated in PVC lures and were observed to be as effective as virgin *C. curvalana* females in trapping studies.

The aldehyde-to-acetate ratio in the sex gland was found to be approximately 1:1, while that of the effluvia was 9:1. This is not unprecedented. In *C. fumiferana* for example, there are large quantities of *E*- and Z11-14:Ac

(95:5) present in the female sex gland and only small quantities of *E*- and Z11-14:Ald (95:5). The reverse is true for the effluvia in which the aldehyde is the major component and the acetate (not designated as a pheromone component) is found in only minor amounts (Silk et al., 1980).

Although tetradecanal was shown to be an EAD-active gland component, its presence in the effluvia was not established. The attractancy of the PVC lures was not affected by its presence or absence. The possibility that tetradecanal is a pheromone component cannot be ruled out at this time.

This work represents the first isolation and identification of pheromone components of a *Croesia* sp. It is unknown whether the components identified here represent the complete pheromone blend of this species. The sex gland extract produced a number of very weak EAD responses in addition to those produced by the components identified in this study. A means for obtaining large numbers of females is required before a search for additional pheromone components can begin; approximately 40 female moths were available for the present study.

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Book Review

Invertebrate-Microbial Interactions: Ingested Fungal Enzymes in Arthropod Biology

M.M. Martin. (1987). Series: Explorations in Chemical Ecology. T. Eisner and J. Meinwald (eds.).

Ithaca and London: Comstock Publishing Associates, 1987. Cloth \$32.50, Paper \$14.95.

In this series, the editors state two objectives, first to bring "the molecular dimension to our understanding of biological relationships, the relationships between animal and plant, the multicellular and unicellular, social and nonsocial, and kin and non-kin." Second, their "purpose is not only to summarize what they have learned but to convey a feeling for what it is like to venture into an unknown area of promise." In Professor Michael Martin, they have found just the author to accomplish their aims.

Invertebrate-Microbial Interactions is too broad a title for this book; its real subject is encapsulated in the subtitle *Ingested Fungal Enzymes in Arthropod Biology*, and the author's summary of his thesis is "I propose that the augmentation of digestive capacity through the ingestion of active fungal or bacterial enzymes may be widespread among invertebrates."

The author demonstrated this thesis in 1978 when he and his wife showed that the fungus-growing termite *Macrotermes natalensis* acquires fungal enzymes from *Termitomyces*, the mutualistic fungus it cultivates, by eating nodules. These cellulolytic enzymes are utilized in the midgut to digest cellulose in the wood the termites eat. In the same year, Abo-Khatuo published an account of a similar process in the related termite *M. subhyalinus*, indicating that the time was ripe for this concept. Subsequently, Martin and his coworkers have gone on to demonstrate sequestered enzymes in the siricid woodwasp *Sirex cyaneus*, and in several cerambycid beetles. They have so far failed to establish their importance in detritivores such as tipulid larvae and woodlice where one might have expected a suite of microbial enzymes to be particularly valuable in the digestion of the more recalcitrant compounds that remain in dead leaves and wood once they are cast off by the plant.

Perhaps the most curious case is the symbiotic relationship between the fungus-growing attine ants, and their symbiotic basidiomycete. Here it appears

that enzymes ingested by the ants (but from exactly what part of the fungus has not been rigorously established) pass right through the gut, where they seem to be unimportant in the ants' digestive process, and emerge in the feces. This was the work in the late sixties and early seventies that stimulated the author's interest in the use of microbial enzymes by arthropods, and yet the explanation seems intuitively to be the least satisfactory. Martin claims that "the feeding and defecation behavior of the workers can be viewed as a mechanism that has replaced the process of enzyme secretion by the fungus." In short, the ants feed on mature fungus garden portions, but defecate in areas where the fungus is just being established, so enhancing the competitive ability and fitness of the fungus. Other workers (Powell, R. J., 1984 *The influence of substrate quality on fungus cultivation by some attine ants*. Ph.D. Thesis, University of Exeter, U.K.) have, however, shown that the fungus is capable of secreting enzymes into its substrate, and it is not clear why other competitive fungi should not find a predigested substrate just as good for rapid growth as the ant fungus itself. It is mysteries like these that make this subject so fascinating and this book such a challenging read.

However, the book is not only an excellent technical account of a specialized area of modern biology, it is a record of a scientist's professional career, what motivated him to take up certain research themes, and how he set about the task. This in its own way is as interesting as the findings and is very frank. It is written in a highly personal way: "I undertook the research . . . with an undisguised preconception about how it would turn out . . . I had convinced myself that the termites were using their fungal symbiont as a source of digestive enzymes . . .," and is a clear demonstration of the quiet revolution that has occurred in the way science is reported (if not necessarily in the way it is conducted). Gone is the use of the passive, impersonal grammar we were instructed to use as students; gone the tenets of the inductivist interpretation of scientific method, where a dispassionate objective scientist observes large numbers of singular examples of biological phenomena, before inducing cautious generalizations that he tests and discards or gradually enlarges to encompass more phenomena. Here we have Popper's falsificationism unashamedly triumphant. "I have been upbraided repeatedly by co-workers, colleagues, students and reviewers for making statements about the general importance of ingested enzymes to arthropod nutrition that are not justified by the comparatively small number of species in which the phenomenon has been rigorously established. I must acknowledge the validity of these criticisms, but am still unable to restrain myself."

Here we catch a glimpse of the genuine scientist with a "bee in his bonnet." I shall use this book with undergraduate students, not only for its technical information and its honest account of the way a research program actually develops, but as a stimulus for seminars on how we should present science and

the extent to which the personalization of issues now so much a feature of political discussion should be allowed to spread to science. At times, my editorial pen twitched convulsively.

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OPTIMAL SEARCH DIRECTION FOR AN ANIMAL FLYING OR SWIMMING IN A WIND OR CURRENT

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Abstract—The problem of the optimal direction in which a flying or swimming animal should search for a chemical plume was addressed. Active spaces were approximated by sphere, prolate ellipsoids, or rectangular parallelepipeds of various length-to-width ratios. The optimum course direction for the sphere was in the direction of flow (downwind). For active spaces that were highly elongated along the direction of the wind or current, the optimal course heading (with respect to the moving medium) was nearly across the flow. For intermediate shapes, the optimal course was intermediate. Because of the effect of the moving medium, these course headings resulted in actual ground tracks that were more in the direction of the flow, depending on the relative speeds of flying (swimming) and the wind (current). When the two speeds were equal, the magnitude of the advantage of choosing the optimum direction over a random direction was close to 50% with a small dependence on the shape of the active space. If the active space was spherical or highly elongated or locomotor speed was much greater than the speed of the current, the advantage approached a factor of $\pi/2$ (≈ 1.57).

Key Words—Search, crosswind, crosscurrent, odor, plume, active space, apparenacy, pheromone.

INTRODUCTION

It has often been asserted that the optimum strategy for an animal searching for a chemical plume is to move across the wind (or current) carrying the plume (e.g., Janzen, 1984). This seems plausible, since a plume has a larger cross section to movement across the wind than parallel to it; undoubtedly this strategy is optimal for animals walking on a substrate in certain circumstances.

However, Sabelis and Schippers (1984) have shown that fluctuations in wind direction can make crosswind searching the least effective direction. Another situation in which crosswind searching is a questionable strategy is for animals flying or swimming in the medium that carries the plume, because they can move faster downwind than crosswind. More specifically one might ask if it is the course heading with respect to the medium that is aimed across the wind or the actual track resulting from the combined effects of wind and locomotion. Another question of interest is how much this matters. How much difference is there on the average between the optimal direction and a randomly chosen direction? This article addresses these questions using simple geometrical shapes for the active space of the plume. Terms will be used that are appropriate for an animal flying in wind to an airborne plume, but the ideas and results apply equally to an animal swimming in a current of water to a waterborne plume.

METHODS AND MATERIALS

The basic assumptions were that the target of the search is stationary with respect to a substrate and the wind is measured with respect to the same substrate, with speed v_w . The animal making the search was assumed to fly at a constant speed with respect to the air, v_a (airspeed), in a straight line with a course heading in any chosen direction, θ , measured from the downwind direction. As a consequence of the wind, in general, the actual track of movement with respect to the substrate has a different direction and speed, v_g (ground speed). In order to detect the target, the searcher must move within a certain volume surrounding the target. This volume is called the active space (Wilson, 1970).

A detailed theory has been worked out for searching for ships at sea (Koopman, 1980). This theory was modified to apply to three dimensions for the problems at hand. Assume identical targets are distributed at random with density, ρ . Each target has a cross-sectional area, σ , perpendicular to the movement of the searcher. The searcher moves with velocity, v_g , in a straight line for a time, t . The advantage of searching in a straight line is discussed elsewhere (Pline and Dusenbery, 1987; Dusenbery, 1989). The probability of finding at least one target is

$$p = 1 - \exp(-\rho\sigma v_g t)$$

The cross section, σ , can represent the cross-sectional area of the active space of a chemical plume around the target or the space defined by the range of detection around the searcher for other types of stimuli. In either case, $S = \sigma v_g t$ is the volume searched. It can be thought of as the volume around the search track in which a target must be located for detection to occur. Search efficiency

is maximized by maximizing the search volume per unit time, $S/t = \sigma v_g$. Thus, the effective search rate is simply the product of the velocity of the searcher with respect to the target times the appropriate cross section. This formulation is consistent with that of Nicholson and Bailey (1935), who treated the effects of searching efficiency of populations on competition. Here we are considering the searching efficiency of individuals—a problem they did not address.

There exists little solid information on the actual shape of chemical plumes under natural conditions because of the ubiquitous occurrence of turbulence in both air and water for size scales relevant to all but microorganisms (Elkinton and Cardé, 1984; Tolmazin, 1985; Murlis, 1986). Consequently, the analysis concentrated on active space shapes that are amenable to mathematical analysis—rectangular parallelepipeds and prolate ellipsoids. Comparison of different shapes was made for active spaces of equal volume.

Spheres. The sphere has the same cross section in all directions, and the volume searched per unit time (search rate) is simply this cross-sectional area times the ground speed. Consequently, the effect of course direction is only to alter the ground speed. For a sphere of radius r , the search rate is

$$S/t = \pi r^2(v_w^2 + v_a^2 + 2v_w v_a \cos \theta)^{1/2}$$

If the searcher flies downwind ($\theta = 0$), the ground speed is simply the sum of the wind and flying speeds, and $S/t = \pi r^2(v_w + v_a)$. This is the maximum search rate because ground speed is maximized. Searching crosswind ($\theta = 90^\circ$) gives $S/t = \pi r^2(v_w^2 + v_a^2)^{1/2}$. For upwind searching, $S/t = \pi r^2(v_w - v_a)$ and, if the speeds are equal, no search is made because there is no net movement. If the two velocities differ greatly from one another, the search rate is simply proportional to the larger velocity, $S/t \approx \pi r^2 v$. This is plausible since, with greatly differing velocities, the direction and speed of the lesser have little influence. The effect of the direction of search is maximum when the two velocities are equal, $v_w = v_a = v$, in which case $S/t = \pi r^2 v(2 + 2 \cos \theta)^{1/2}$. By integrating over all directions, it can be determined that the average search rate for randomly chosen directions is $\langle S/t \rangle = 4r^2 v$. The ratio of optimum to random search rates is thus $\pi/2 \approx 1.5708$, under the assumption of equal speeds.

Chemical plumes in nature are elongated downwind, but their shape is poorly defined. They were represented by two types of shapes: rectangular parallelepipeds permit relatively simple calculations, while prolate ellipsoids probably provide a more realistic representation of actual active spaces. The height (H) and width (W) were assumed equal and perpendicular to the wind. The search was confined to the plane containing the width and length (L), and perpendicular to the height.

Rectangular Parallelepipeds. For these the cross section for crosswind movement is height times length, $\sigma = HL$, while that for up- or downwind movement is height times width, $\sigma = HW$. The maximum cross section is the

height times the length/width diagonal, $\sigma = H(L^2 + W^2)^{1/2}$. With the rectangular geometry parallel to the wind, the volume searched is simply the sum of two parts. One is the cross section perpendicular to the wind times the component of ground velocity parallel to the wind. The other part is the cross section parallel to the wind times the component of ground velocity perpendicular to the wind. Thus,

$$S/t = v_w HW + v_a H(W \cos \theta + L \sin \theta)$$

If flying speed is much greater than windspeed, the first term drops out. Conversely, if windspeed is much greater than flying speed, $S/t \approx v_w HW$. For flight in the downwind direction, the two velocities sum and $S/t = HW(v_w + v_a)$. For upwind flight, it is the difference rather than the sum that is proportional to search rate. Flying crosswind results in $S/t = v_w HW + v_a HL$. The average search rate for randomly chosen directions can be determined by integrating over all directions to obtain $\langle S/t \rangle = v_w HW + (2/\pi) v_a HL$. The optimum search direction is $\theta = \arctan(L/W)$. A search in this direction has a rate of $S/t = v_w HW + v_a H(W^2 + L^2)^{1/2}$. If $L \gg W$ and $v_a \approx v_w$, the ratio of optimal to random search rates simplifies to $\pi/2$.

Prolate Ellipsoid. For an ellipsoid with semiaxes a , b , and c , a prolate ellipsoid is defined by $a > b = c$. (A sphere has $a = b = c$.) It will be assumed that the major semiaxis, a , is parallel to the wind direction. The length is twice the major semiaxis, $L = 2a$, and the height and width are twice either of the minor semiaxes, $H = W = 2b = 2c$. The axial ratio is $a/b = L/W$. It can be shown that the projected area in any direction, θ , measured from the major axis, a , and perpendicular to c gives a cross section, $\sigma(\theta) = \pi c(a^2 \sin^2 \theta + b^2 \cos^2 \theta)^{1/2}$. The search rate for any track direction is the product of the appropriate cross section and ground speed. Integration of the search rate over all directions to determine the average search rate for random directions and determination of the direction of maximum search rate were performed using numerical approximations on a computer.

RESULTS

In order to make quantitative comparisons, flying speed was assumed equal to windspeed, which maximizes the importance of search direction. These speeds were assumed to have a numerical value of 1 in the units used (e.g., m/s). An additional assumption was that the different-shaped plumes all had the same volume equal to 1 (e.g., 1 m^3). Since search rate is simply related to speed of movement and size of plume, the data presented can easily be scaled to fit other values for speed or volume.

Basic data for rectangular active spaces of various axial ratios are presented in Table 1. Similar data for ellipsoids are presented in Table 2.

TABLE 1. DATA FOR RECTANGULAR SHAPES

Active space				
Axial Ratio	1	10	100	1000
Width	1.000	0.464	0.215	0.100
Height	1.000	0.464	0.215	0.100
Length	1.000	4.642	21.544	100.000
Volume	1.000	1.000	1.000	1.000
Optimum angle to wind (°)				
Heading	45	84.3	89.43	89.943
Track	22.5	42.14	44.71	44.971
Search rate				
Optimum	2.414	2.381	4.688	10.010
Random	1.637	1.587	3.001	6.376
Ratio	1.475	1.500	1.562	1.570

The optimum course angle to the wind is 0 for the sphere and increases toward 90° as the active space becomes more elongated. Figure 1 shows the relationship between search rate and the full range of course headings for five ellipsoids or varying axial ratio. In each case, the search rate goes to 0 for an upwind course ($\theta = 180^\circ$) because of the assumption of equal wind and flying speeds. The figure shows that the efficiency of a downwind search ($\theta = 0^\circ$) depends strongly on the shape of the active space; for a sphere it is the optimal direction but, as the active space elongates, the efficiency diminishes sharply.

The tables also show the angles the resulting tracks make under the influ-

TABLE 2. DATA FOR ELLIPSOIDAL SHAPES

Active space					
Axial ratio	1 ^a	2	5	10	100
Width	1.241	0.985	0.726	0.576	0.267
Height	1.241	0.985	0.726	0.576	0.267
Length	1.241	1.969	3.628	5.759	26.730
Volume	1.000	1.000	1.000	1.000	1.000
Optimum angle to wind (°)					
Heading	0	70.529	87.612	89.421	89.994
Track	0	35.264	43.806	44.711	44.997
Search rate					
Optimum	2.418	1.759	2.110	2.618	5.612
Random	1.539	1.338	1.439	1.708	3.574
Ratio	1.571	1.314	1.466	1.533	1.570

^aThe ellipsoid with axial ratio of 1 is a sphere.

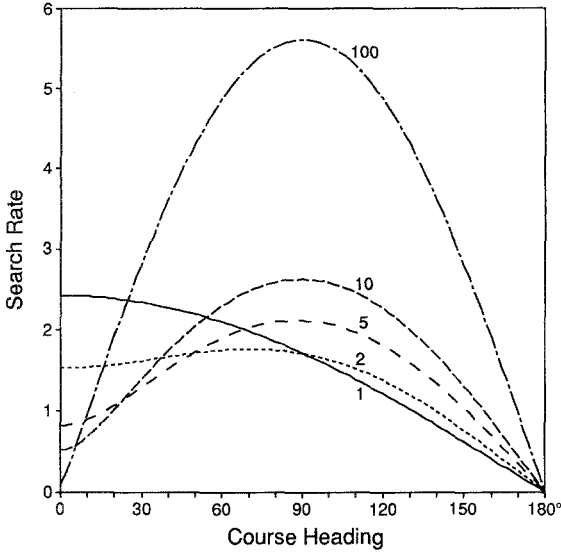


FIG. 1. Search rate as a function of course heading. Search rate is the volume searched per unit time. Active spaces are prolate ellipsoids of equal volume with axial ratios of 1, 2, 5, 10, and 100 as indicated.

ence of the wind. With the assumptions of equal wind and flying speeds, the tracks were directed just half as far from the downwind direction as is the course heading.

The search rates for optimum and random course headings are also presented in the tables. For each type of heading, the search rate varies little from isometric shapes out to axial ratios of about 10 but increases as the active space is elongated further. This pattern can be understood from the more detailed data on rectangular active shapes (Figure 2). The search rate actually goes through a minimum for L/W ratios of about 3, and then increases steadily, approaching a relation of $S/t \propto (L/W)^{1/3}$.

The relative advantage of choosing the optimum course heading can be determined from the ratio of optimum to random search rates. The data of the table show this ratio to vary in a narrow range around 1.5. The sphere has the highest value of $\pi/2 \approx 1.5708$. The pattern for rectangular spaces is shown in more detail in Figure 3. It is clear that the ratio goes through a minimum at $L/W \approx 2$ and then increases toward a plateau value of $\pi/2$, the same as the value for the sphere. Table 2 shows that ellipsoidal shapes give a similar pattern.

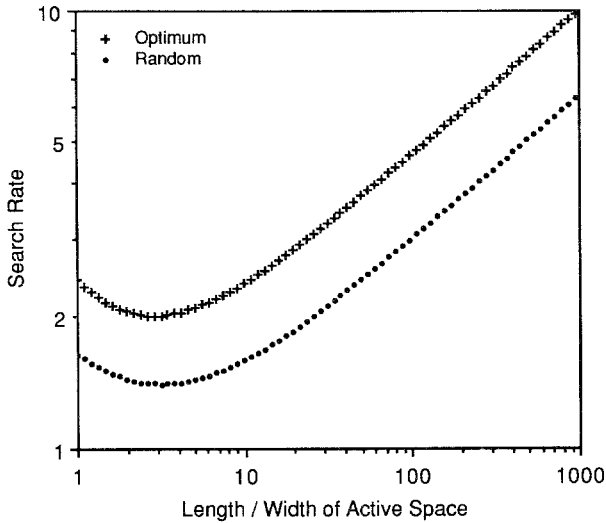


FIG. 2. Search rate as a function of length-to-width ratio of rectangular active spaces.

DISCUSSION

The question of the optimum direction for searching in wind for a chemical plume can now be answered, at least for certain situations. For a spherical active space, the optimum direction is downwind. As the active space elongates downwind, the optimal course heading swings toward a crosswind direction. Although there always remains a slight downwind bias, this would probably not be observable for plumes with axial ratios of 10 or more. There is at least some evidence that some animals search downwind (Gillies and Wilkes, 1974).

The data also clearly demonstrate that a longer plume of a given volume has greater apparency. However, it should be pointed out that a simple increase in wind velocity does not generally produce a more elongated plume. In the model of Bossert and Wilson (1963), an increase in wind velocity increases turbulent mixing, which actually decreases the volume of the active space without much change in proportions. If diffusion is assumed constant, the length of a plume is independent of wind speed (Roberts, 1923).

The question of the magnitude of the advantage of choosing the optimum course direction can be answered fairly precisely. From the data of the tables and Figure 3, it is clear that the advantage depends on the shape of the active space. However, the variation is confined to a small range, with ratios between 1.3 and 1.58. Thus, it can be said that the optimal search direction is about

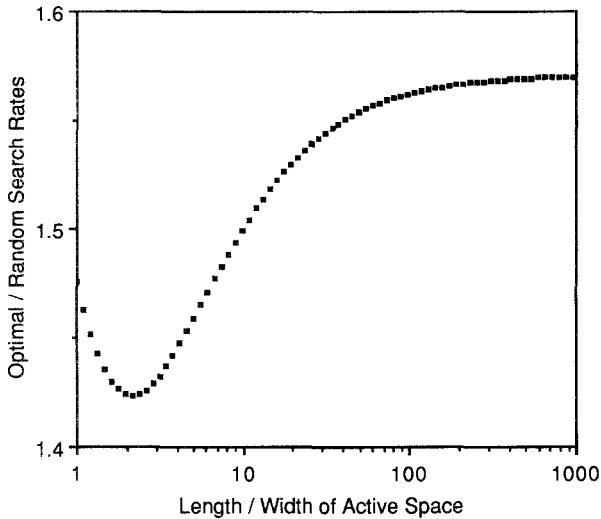


FIG. 3. Ratio (of the search rate for the optimal direction of search to the average search rate for random directions) as a function of length-to-width ratio of rectangular active spaces.

50% more efficient than a randomly chosen direction. In many situations of interest, flying speed is greater than wind speed and the plume is elongated. This leads to an optimum/random ratio of $\pi/2$, corresponding to an increase in efficiency of 57%.

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QUINOLIZIDINE ALKALOIDS OBTAINED BY *Pedicularis semibarbata* (SCROPHULARIACEAE) FROM *Lupinus fulcratus* (LEGUMINOSAE) FAIL TO INFLUENCE THE SPECIALIST HERBIVORE *Euphydryas editha* (LEPIDOPTERA)

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Abstract—*Pedicularis semibarbata* is apparently an obligate hemiparasite of coniferous trees. It is also a facultative parasite of *Lupinus fulcratus* from which we find that it obtains quinolizidine alkaloids, principally α -isolupanine. As a result, a single population of *P. semibarbata* contains both alkaloid-rich and alkaloid-free plants. The butterfly *Euphydryas editha* naturally oviposits on both plant types. This butterfly population, which is the principal herbivore attacking *P. semibarbata* at this site, is known to contain two morphs. Individuals of a specialist morph discriminate when ovipositing among individual *P. semibarbata* plants and produce offspring that survive better on accepted than on rejected plants. Those of a generalist morph accept all *P. semibarbata* plants and produce offspring that survive equally well on plants accepted or rejected by the discriminating morph. Because of the existence of this complex variation among the butterflies, the presence of naturally laid eggs on alkaloid-containing plants still leaves the possibility that the alkaloids may defend the plants against the specialist morph. In experiments on both oviposition preference and larval performance in early instars, we failed to detect any correlation between alkaloid content of a plant and either its acceptability to or suitability for the discriminating morph of the insect. Alkaloid presence in the host-plant population, achieved through root parasitism, is currently neither subject to strong insect-mediated selection nor a major cause of selection on the insects.

Key Words—*Pedicularis semibarbata*, Scrophulariaceae, *Lupinus fulcratus*, Fabaceae, *Euphydryas editha*, Lepidoptera, Nymphalidae, herbivory, quinolizidine alkaloids, isolupanine, oviposition, parasitic plants.

INTRODUCTION

Harris and Stermitz (1987) showed that root parasitic *Castilleja* species acquire quinolizidine alkaloids from their hosts. This class of alkaloid has been implicated several times in the allelochemical defense of plants (Dolinger et al., 1973; Bentley et al., 1984; Wink, 1985; for example). A single population of parasitic *Castilleja* was found to contain both plants with alkaloids and plants without them. Here, we report a similar finding for a population of the hemiparasite *Pedicularis semibarbata* Gray (Scrophulariaceae) that also contains both alkaloid-rich and alkaloid-free individuals. Such plant populations provide natural systems in which correlations between alkaloid concentration and herbivore preference can be studied, with implications for the importance of secondary utilization by hemiparasites of host defensive compounds (Atsatt, 1977). For these purposes we explored the interaction between *P. semibarbata* and *Euphydryas editha* Bdv (Lepidoptera: Nymphalidae).

METHODS AND MATERIALS

Study Site. Our study site was at Rabbit Meadow along the Generals' Highway above Fresno in Sequoia National Forest, California. At this site the distribution of *E. editha* eggs among four host-plant species has been described by Singer (1983), who found that *P. semibarbata* received about 70% of the eggs deposited by the insect population. Search behavior of the insects for *P. semibarbata* has been studied by Rausher et al. (1981) and by Mackay (1985).

Plant Chemistry. *Lupinus fulcratus* Greene was identified by D. Wilken, Department of Biology, Colorado State University and a voucher (FRS 326) deposited in the Colorado State University Herbarium. The alkaloid content of *L. fulcratus* and its parasitic *P. semibarbata* was determined as follows.

For a typical alkaloid isolation, 1.3 g of *L. fulcratus* was crushed, wetted with 5% NaHCO₃ and stirred in 55 ml of 1:1 butanol-toluene for 48 hr. The mixture was filtered and extracted three times with 10 ml of 1 M H₂SO₄. The combined acid layers were extracted with CHCl₃ and made basic to pH 9 with NaOH pellets. The basic solution was extracted three times with equal volumes of CHCl₃, the CHCl₃ layers combined, and evaporated to yield 34 mg of alkaloid mixture.

Essentially the same procedure was used for semiquantitative alkaloid analysis of individual *P. semibarbata* plants. Samples of either 100 or 50 mg

of dry *P. semibarbata* plants from a 1986 collection were extracted and the final CHCl_3 solutions evaporated in test tubes. To each tube was added 1.0 ml of CHCl_3 . Then, 0.10 ml for the 100-mg samples or 0.20 ml for the 50-mg samples was applied to Si gel (0.25 mm, Merck 60) TLC plates. The plates were developed in 9:1 CHCl_3 -MeOH and spots visualized with iodoplatinic acid spray. Spot sizes and intensities were visually compared. Five 100-mg samples of individual *L. fulcratus* plants were treated and analyzed similarly. Alkaloid presence was readily detected qualitatively using 5–10 mg of dry small individual *P. semibarbata* leaf samples with a modified procedure. Here, the leaf was macerated in a drop or two of 1 M Na_2CO_3 solution in the bottom of a small test tube and about 0.5 ml of 2:1 CHCl_3 -MeOH added. The mixture was agitated with a small stirring rod for a few minutes, allowed to stand briefly, and the CHCl_3 was drawn off with a disposable pipet into a depression in a spot plate. The CHCl_3 was allowed to evaporate to a small volume and one tenth to one half spotted on a TLC plate, then developed and visualized as above. This procedure is easily adapted to use in the field.

Alkaloid residues from the 1986 1.3 g *L. fulcratus* extraction and the combined *P. semibarbata* extractions were analyzed by NH_3 chemical ionization mass spectrometry, ^{13}C NMR spectroscopy, and TLC.

Insect Preferences. Insect preference was tested by placing field-caught insects on undisturbed plants still growing in their natural positions, using the technique of Singer (1986). If deprived of the opportunity to oviposit, discriminating insects will consistently accept some individual plants and reject others.

We performed two experiments in separate years, 1986 and 1988. In 1986 we tested randomly selected morphologically similar plants with no initial knowledge of their alkaloid content. By this means we obtained pairs of accepted and rejected plants. We then analyzed the plants for alkaloids. Only in those cases where alkaloid concentrations were different in accepted and rejected members of a pair could we obtain information about the correlation between alkaloid content and acceptability. In order to increase our efficiency in the second experiment in 1988, we assessed alkaloid content first, then paired each plant that contained alkaloids with a morphologically similar plant that was alkaloid-free. Persons who performed preference trials to determine which (if any) member of the plant pair was more acceptable were ignorant of the identities of the plants that actually contained alkaloids.

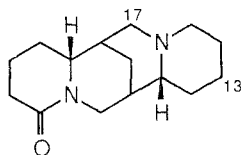
Insect Performance. Eggs of *E. editha* are laid in clusters and larvae are gregarious, occurring naturally in groups of 5–100. As each egg cluster hatched, two groups of 20 newly hatched larvae were isolated. One group of 20 was placed on each member of a plant pair (still growing *in situ*) and allowed to feed for 10 days, after which the remaining larvae were gathered and counted. Again, two experiments were performed in separate years, but with some differences between the years. In 1986, we used eggs gathered at random and

performed alkaloid analyses after larval feeding, while in 1988 we used offspring of discriminating butterflies and performed the analyses before the experiment.

RESULTS

Alkaloid Content. TLC on *L. fulcratus* and 16 of 40 individual *P. semi-barbata* plants from the 1986 experiment showed the presence of one major alkaloid at R_f 0.40 along with a small amount of a second at R_f 0.05. NH_3 chemical ionization mass spectra of the alkaloid fractions from both plants were nearly identical and showed a very large $M+1$ ion at m/z 249 and a very small one at 263, indicating the presence of one major and one very minor alkaloid of mol wt 248 and 262, respectively. The mass and [^{13}C]NMR spectra and TLC of the *L. fulcratus* extract corresponded to data for α -isolupanine (Scheme 1), which we had previously isolated (Harris and Stermitz, 1987; Harris, 1987) and identified from *Castilleja miniata* (GH 167, Emerald Lake; Harris, 1987) and its host, *L. argenteus* subsp. *spathulatus*. The molecular weight 262 corresponds to that of 17-oxoisolupanine (or an isomer; Kinghorn and Balandrin, 1984), but no attempt was made to further identify this minor component.

Alkaloid Content and Butterfly Oviposition Behavior. In the 1986 experiment, alkaloid analyses were performed on 20 previously identified pairs of accepted and rejected plants. Table 1 shows that eight pairs contained no alkaloids in either plant and three contained alkaloids in both members. This left nine pairs in which one member contained alkaloids while the other did not. Of these, six pairs had alkaloids in the rejected plant and three in the accepted plant. Among the three pairs that both contained alkaloids, the concentrations were about equal in both plants of one pair and greater in the rejected members of the other two pairs. So, in total, we found eight pairs with more alkaloids in the rejected than in the accepted plant and three with more in the accepted than in the rejected. If alkaloid content is independent of acceptability, the probability of observing pairs in which the accepted plant contained more alkaloids than the rejected plant should be equal to the probability of observing pairs in



α -isolupanine

SCHEME 1.

TABLE 1. SURVIVAL OF TWENTY *Euphydryas editha* LARVA ON ALKALOID-CONTAINING AND ALKALOID-FREE PLANTS OF *Pedicularis semibarbata* TESTED FOR OVIPOSITION.

Plant pair:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Accepted:	7 ^a	0 ^b	9	16	12	7	14	14	17	11	0	11	0	0	2	17	17	10	18	13
Rejected:	14	0	14	18	13	10	11	12	0	7	0	8	6	0	9	10	4	3	8	13

Larval survival on 15 plants with alkaloids: $X = 8.2$; S.D. = 6.39
 Larval survival on 25 plants without alkaloids: $X = 9.28$; S.D. = 5.7

^aNormal type: alkaloid-free.

^bBold face: alkaloid-containing.

which the accepted plant contained less alkaloids than the rejected plant (0.50 each). With the finding of eight pairs of plants with more alkaloids in the rejected member and three pairs of plants with more alkaloids in the accepted member, we cannot reject this null hypothesis (Sign test, $Z = 1.21$, $P > 0.10$). In the 1988 experiment, where the alkaloid content of the plants was identified first, the acceptabilities of 15 pairs of plants with and without alkaloids were compared. No difference in acceptability was found between alkaloid-containing and alkaloid-free members in two of the 15 pairs. The alkaloid-containing member was preferred in eight pairs, while the alkaloid-free member was preferred in five pairs (Sign test, $Z = 0.55$, $P > 0.10$).

Alkaloid Content and Early Larval Survival. The number of larvae, out of each grouping of 20, that survived to 10 days is also given in Table 1. Overall, there is no significant difference in larval survival on alkaloid-containing versus alkaloid-free plants ($t = 0.554$, $df = 38$, $P > 0.25$). In this and other studies at Generals' Highway, a number of cases are always observed where there is no survival. Small larval webs are set up initially, but the larvae disappear after only one or two days. From observations, such young larvae are not able to survive away from web or the plant, so their disappearance is considered as mortality. We cannot, however, attribute this mortality specifically to a plant effect since the actual cause is not known. If the cases with zero larval survival are eliminated, the average number of larvae surviving (out of 20 placed) is 11.8 (SD = 4.53, $N = 11$) on alkaloid-containing plants, and 10.1 (SD = 4.97, $N = 21$) on alkaloid-free plants (not significantly different; $t = 0.61$, $df = 30$, $P > 0.25$). There is also no significant difference in larval survival between alkaloid-containing and alkaloid-free members of paired plants (average paired difference -2.09 , SD = 6.66, $t = 1.04$, $df = 1$, $P > 0.25$).

In 1988 we observed higher larval survival on the alkaloid-containing plant in five pairs, higher survival on the alkaloid-free member in five pairs, and equal survival on both plants in a single pair. Because there is no trend at all in these data, statistical analysis is not necessary.

DISCUSSION

Plant Parasitism and Alkaloid Transfer. With this work, the transfer of alkaloids from a host plant to a normally alkaloid-free root parasite has now been established for *Pedicularis*, extending the results we found for *Castilleja* (Harris and Stermitz, 1987). As in the *Castilleja* case, root parasitism on a *Lupinus* species has resulted in quinolizidine alkaloid transfer. The quinolizidine alkaloid *N*-methycytisine was previously reported (Ubaev et al., 1963) to be present in *P. olgae*. Our finding of isolupanine as a transferred alkaloid in *P. semibarbata* adds weight to the suggestion (Harris and Stermitz, 1987) that

the *N*-methycytisine in *P. olgae* was probably also the result of root parasitism on a quinolizidine-containing host plant.

P. semibarbata appears from its distribution to be an obligate associate of conifers. This hypothesis is supported by the observation that, whenever trees are removed by clear-cutting, the *Pedicularis* invariably die in one to two years, even though many of them must also be parasitizing *L. fulcratus*, which is not killed by logging. The deaths of *P. semibarbata* after logging do not result from increased insolation, since this plant often grows in open habitats, provided that conifers are close by. In contrast to the obligate association with conifers, many *P. semibarbata* are much too distant from *L. fulcratus* for the possibility of root contact, so the association of the parasite with this plant must be facultative. It appears, then, that *P. semibarbata* requires conifers for survival, but cannot survive solely as a parasite of *L. fulcratus*, from which it nonetheless acquires alkaloids.

Oviposition Behavior. Ng (1988) showed that, with respect to *P. semibarbata*, all discriminating female *E. editha* accept and reject the same plants. Individual plants retain their accepted or rejected status from year to year. Plants that are accepted in preference trials receive more naturally laid eggs than rejected plants (Ng, 1987). We were interested in learning whether or not the acquisition of alkaloids was relevant to the correlated variation of insect adult preference and larval performance shown by Ng (1988). With respect to preference, nine pairs of accepted/rejected plants in our 1986 experiment showed no difference within the pair in alkaloid concentration. For pairs in which there was such a difference, the nonsignificant correlation between alkaloids and host acceptability found in 1986 was in the opposite direction to the even less significant trend in 1988. We conclude that any correlation between alkaloid content and acceptability that may exist could at best explain only a small proportion of the variation in plant acceptability. It could not be the basis of the discrimination phenomena demonstrated by Ng, the chemical basis of which remains unsolved. Ng (1987) showed that variation of water content and nitrogen content were likewise not correlated with acceptability of *P. semibarbata* in the field.

Early Larval Survival. In 1986 we found a nonsignificant trend for better survival on the alkaloid-containing plants and in 1988 found no trend at all in the data. Our conclusion is that the quinolizidine alkaloid content (principally α -isolupanine) of *P. semibarbata* does not render it toxic to young *E. editha* larvae at General's Highway even if they are the offspring of discriminating mothers. We do not think it likely that toxic effects would have been found if we had used later development stages, since early instar lepidopteran larvae are usually more sensitive than later instars to host-plant quality (e.g. White, 1978; Harrison and Karban, 1986; Johnson and Bentley, 1988).

Our sample sizes (20 plant pairs in 1986 and 11 in 1988) are not large,

and a larger data set with more power to detect small correlations between alkaloid content and host-plant acceptability or suitability would be advantageous. Such a data set would, however, be difficult to obtain because the season is short (four weeks) and the work of establishing accepted and rejected plant pairs is labor-intensive.

Context. Previous assessments of insect herbivore interactions with quinolizidine alkaloids or quinolizidine-containing plants have encompassed a variety of situations. Most recently, the lupine alkaloids lupanine and sparteine were shown to reduce growth and survival of first instars of the generalist *Spodoptera eridamia*, but not the growth of later instars (Johnson and Bentley, 1988). Extensive work on quinolizidine alkaloids and "sweet" (alkaloid-free) or "bitter" *Lupinus* species (Wink and Römer, 1986; Wink, 1985, 1987 and references therein) has shown deterrence of leaf miner and aphid generalist feeding and the use of sequestered alkaloids as toxic defenses by specialist aphids. Others have also shown quinolizidine alkaloid deterrence to aphids (Dreyer et al., 1985) and to spruce budworm (Bentley et al., 1984) feeding. In some of these cases, it was clearly evident that there was both herbivore taxon and quinolizidine structure dependence on bioactivity, but even here consistency has not always been observed. For example, 13-acyllupanines were highly deterrent to spruce budworm (*Choristoneura fumifera*) larval feeding, but several other quinolizidines, including α -isolupanine, were not (Bentley et al., 1984), at least at the concentrations used. In contrast, *Lupinus floribundus*, the most heavily attacked of three lupine species studied at several sites (Dolinger et al., 1973) had relatively high concentrations of 13-acyllupanines, which were lacking in the less attacked species. The herbivores, *Glaucopsyche lygdamus* lycaenid butterfly larvae, were also reported to have higher survival in feeding studies on the 13-acyllupanine containing *L. floribundus* than on those species containing nonester quinolizidines.

The much-cited work of Dolinger et al. (1973) was perhaps the closest to our own work in aim. These authors were the first to suggest that interindividual variability of host-plant quinolizidine alkaloid content may have evolved in response to frequency-dependent selection exerted by insects. Working at several high elevation sites in Gunnison County, Colorado, both Dolinger et al. (1973) and Breedlove and Ehrlich (1972) searched for correlations between traits of different lupine species and oviposition by *G. lygdamus*. The plant traits investigated were different in the two studies; Breedlove and Ehrlich used pubescence and Dolinger et al. used alkaloid content. Breedlove and Ehrlich concluded that differences among attack rates on three lupine species were related to differences in pubescence among these species. Dolinger et al. (without discussing the prior results of Breedlove and Ehrlich) concluded that the same differences were related to "alkaloid configuration." Neither conclusion appears to be justified, because both the correlation shown by Breedlove and

Ehrlich between insect attack and pubescence and the correlation shown by Dolinger et al. between insect attack (or host availability) and alkaloid content fall far short of statistical significance. However, the work of Dolinger et al. does show indisputably that accumulation of high quantities of a complex mixture of quinolizidine alkaloids, some of which were subsequently shown to be highly toxic to a generalist (Bentley et al., 1984), is not sufficient to deter extensive attack by a specialist.

Some of the difficulties encountered by Dolinger et al. stemmed from attempts to make comparisons simultaneously across species and populations. In our work, we have attempted to reduce problems of data interpretation by estimating traits within, rather than among, plant and insect populations. In our *P. semibarbata* and *E. editha* system, the conclusion is that alkaloid concentration, although highly variable, is currently neither subject to strong insect-mediated selection nor a major cause of selection on the insects. We earlier identified *Castilleja* species and have now found additional *Pedicularis* species that accumulate other, perhaps more deterrent or toxic, quinolizidine or pyrrolizidine alkaloids. These populations also contain alkaloid-free plants, are attacked by insects, and should provide additional systems for study.

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CONSPECIFIC SCENT TRAILING BY GARTER SNAKES
(*Thamnophis sirtalis*) DURING AUTUMN
Further Evidence for Use of Pheromones in Den Location

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Abstract—Adult garter snakes (*Thamnophis sirtalis*), collected in October near a traditional, communal hibernaculum in central Wisconsin, were acclimated to autumnal conditions and subjected to laboratory tests to determine whether they could follow scent trails of a conspecific. Graded responses were obtained, but 75% of the sample showed at least some inclination to follow scent trails. The results suggest that pheromone cues may be used by male and female garter snakes to locate traditional dens during autumnal migrations. Evidence from this and other studies suggests that pheromones are probably used in conjunction with other homing mechanisms and that the role of pheromones in den location may be more important in younger snakes and in populations inhabiting northern latitudes.

Key Words—Pheromone, scent trail, chemical cue, hibernation, denning, homing, garter snake, *Thamnophis sirtalis*, Colubridae.

INTRODUCTION

An increasing literature concerning the role of chemical signals in the socio-biology of snakes (see Ford, 1986, for a recent review) has shown that the vomeronasal sense of these organisms is important and highly developed. Most studies of snake pheromones have addressed reproductive behaviors; however, scent trailing of conspecifics has been implicated in the formation of nonsexual aggregations for the purpose of avoiding unfavorable environmental conditions and reducing predation (e.g., Dundee and Miller, 1968; Duvall et al., 1985; Graves et al., 1986; Noble and Clausen, 1936). Additionally, chemical cues

have long been suspected to play a part in the relocation of communal snake dens during autumn (Hirth, 1966; Klauber, 1956; Noble and Clausen, 1936), but few investigators have attempted rigorous tests of this hypothesis.

In northern climates, strong selection pressures have likely played a major role in the evolutionary development of mechanisms by which snakes relocate traditional dens during autumnal migrations from (often distant) summer habitats. Hibernacula with features that effectively protect snakes from severe winter conditions may be limited on a local basis; in fact, this may be a reason why snakes den communally (Gregory, 1984). Snakes unable to relocate "proven" dens are likely subject to greater risks of mortality during winter. Also, mating opportunities in northern snake populations are often restricted to the den site (Gregory, 1982, 1984); thus, any individual not in attendance may forfeit its chance to contribute to the genome. Not unexpectedly, long autumnal migrations (up to 17.7 km in *T. sirtalis*; Gregory, 1984) to communal dens are made by many species and, in numerous cases, individuals show remarkably high fidelity to particular hibernacula (Gregory, 1982, 1984).

Evidence that snakes are able to follow scent trails of conspecifics has been documented for 24 species representing five families (Ford, 1986). Few studies, however, have addressed this behavior in the context of den relocation (Brown and MacLean, 1983; Graves et al., 1986; Lawson, 1985). Accordingly, this study was undertaken to assess the ability of adult garter snakes, from a communally denning population in Wisconsin, to follow conspecific scent trails under simulated autumnal conditions.

METHODS AND MATERIALS

Eastern garter snakes (*Thamnophis sirtalis*) were collected during October 1986 as they gathered near a traditional, communal den in central Wisconsin (described by Costanzo, 1986). Twenty-eight snakes were housed, irrespective of body size or sex, in small groups. One additional snake, a large (94 g) female designated for use in forming scent trails, was maintained separately. Snakes were kept in plastic boxes and were exposed to autumnal circadian thermal and light regimes (20°C, 13 hr photophase; 5°C, 11 hr scotophase) for 14 days prior to testing. Snakes were provided with water, but food was withheld.

Trailing ability was tested (procedure modified after Ford, 1981) during the last week in October, under photophase conditions (overhead fluorescent lighting; 387 lux), in a rectangular, plywood arena (Figure 1). Seventeen nails spaced at 5.5-cm intervals protruded from the floor and formed a continuous series of gates through which a snake could pass. The nails were arranged in a slight arc so that all gates were located 200 cm from an entrance port in the front wall of the arena. The arena surface was covered with corrugated paper

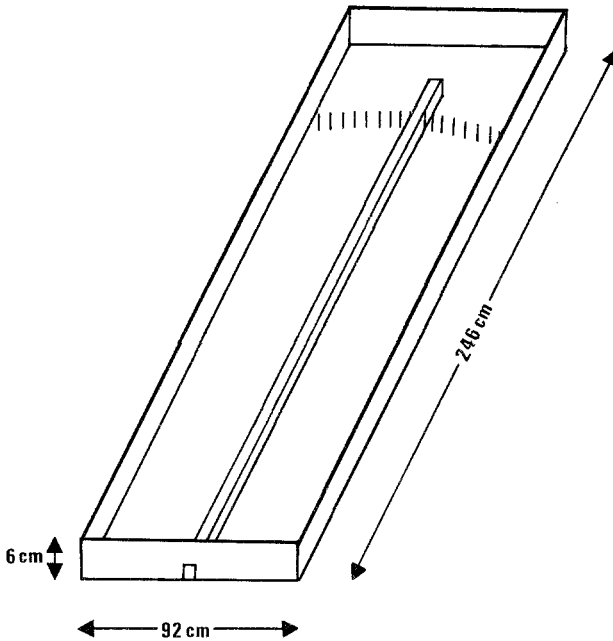


FIG. 1. Diagram of the testing arena used to measure the trail following ability of garter snakes during autumn. Scent trails were produced by guiding the movement of a stimulus snake, which was confined inside a bottomless, removable tunnel (shown), down the length of the arena and through a randomly chosen gate.

upon which 1200 ml of unscented cat litter (clay particles) was dispersed to provide traction. Nails were covered with cocktail straws to protect them from scent contamination. All coverings were replaced after each trailing and control test to ensure that residual odors were removed.

To promote stimulus uniformity, only one animal was used to create scent trails; fresh trails were produced within 30 min of testing each subject for trailing ability. This was done by guiding the movements of the large female snake, which was enclosed inside a 4.5-cm wide, bottomless tunnel, down the length of the arena and through a gate (Figure 1). The tunnel and snake were removed prior to introducing the experimental subject. Gates containing scent were chosen randomly, except that the three nearest each side wall were never used; this restriction was necessary since pilot studies and Ford (1986) indicated that non-trailing and control snakes had a strong affinity for arena walls.

Prior to testing, each subject was habituated for 20 min inside a holding box at the front of the arena. A pulley system was used to remove a partition between the holding box and the entrance port, thus allowing the snake to enter

the arena. Trials involving snakes not entering the arena within 15 min were terminated. Movements and gate choices of snakes were observed from behind a blind 2.5 m away. After a subject passed completely through a gate and reached the distal wall of the arena, it was replaced in its holding cage. Snakes were tested in control situations (no scent trail present) four to six days later and then sexed.

RESULTS

Twenty-four of 28 snakes completed the control trials. All but one individual (96%) passed through end gates (the two adjacent gates nearest each side wall), thus, "wall-seeking" was a prominent behavioral characteristic of non-trailing snakes. This necessitated statistical evaluation of the results using a binomial test (choice of four end gates vs. choice of 14 middle gates; Sokal and Rohlf, 1969). Frequencies recorded for control snakes were significantly different from probabilities based upon random gate choice (Table 1). Binomial probabilities calculated for experimental and control groups showed that the presence of conspecific scent in the arena significantly influenced snake behavior (Table 1).

Of the 28 snakes completing experimental (trials present) trails, 75% showed at least some inclination to follow scent trails. Trailing snakes demonstrated a behavior similar to the trail contact response (TCR) (Brown and MacLean, 1983), which involved slow and methodical sampling of the substrate, over which the trail-forming snake had passed, with its tongue tips. This behavior, which did not occur in control tests, allowed the observer to estimate (to within 10 cm) the length of the trail followed.

Garter snakes showed differential responses to the presence of conspecific

TABLE 1. EXPECTED AND OBSERVED BINOMIAL PROBABILITIES FOR GATE CHOICES OF GARTER SNAKES TESTED IN LABORATORY ARENA FOR ABILITY TO FOLLOW CONSPECIFIC SCENT TRAILS DURING AUTUMN

Test condition	Probability of end gate choice	Probability of middle gate choice	N	Significance (P)
Expected	0.222	0.778		
Control	0.958	0.042	24	<0.005 ^a
Experimental	0.607	0.393	28	<0.005 ^b

^aProbability that responses of snakes tested in the absence of scent trails do not differ from random gate selection.

^bProbability that responses of snakes tested in the presence of scent trails do not differ from those obtained in control tests.

scent in the test arena. Strong trailing inclination was demonstrated by seven snakes (25%) that correctly chose the scented gate and four snakes (14%) that chose a gate immediately adjacent to the scented one. Ten snakes (36%) trailed initially (mean distance \pm SEM = 74 ± 15 cm), but eventually passed through end gates. Seven individuals (25%) showed no inclination to trail and also chose end gates. Snakes were classified, based on their inclination to trail, as exact trailers, near-exact trailers, partial trailers, or nontrailers (Table 2). Relative to males, a greater proportion of females (75%) showed partial and nontraining responses, but contingency analysis (Sokal and Rohlf, 1969) indicated that this difference was nonsignificant (chi square = 1.79, $df = 1$, $P > 0.20$).

DISCUSSION

The present study showed that garter snakes of both sexes, acclimated and tested under autumnal conditions, can follow the scent trails of a conspecific. This result concurs with the field observation that *T. sirtalis* followed common and well-defined "travel lanes" in their approach to the den (Costanzo, 1986, 1988) and further supports the hypothesis that pheromones are used for den location by snakes in nature.

The present study is the first to provide direct experimental evidence for trail following, in the context of den location, for any colubrid snake. However, Brown and MacLean (1983) convincingly demonstrated that neonate timber rattlesnakes (*Crotalus horridus*) from New York followed the trails of litter mates and adults of both sexes during autumn. This behavior apparently was not demonstrated by congeneric (*C. viridis*) adults from Wyoming, although details of testing protocol and specific results were not reported (King et al., 1983). Graves et al. (1986) later showed that *C. viridis* neonates were attracted to scents depos-

TABLE 2. PERFORMANCE, SEX RATIO, AND MASS DATA FOR GARTER SNAKES TESTED IN LABORATORY ARENA FOR ABILITY TO FOLLOW CONSPECIFIC SCENT TRAILS DURING AUTUMN

Performance group ^a	N	Males (N)	Females (N)	Mass ($\bar{X} \pm$ SEM)
Exact trailers	7	6	1	38.3 \pm 5.7
Near-exact trailers	4	2	2	26.3 \pm 6.6
Partial trailers	10	6	4	39.8 \pm 3.7
Nontrailers	7	2	5	62.9 \pm 10.0

^aSnakes were assigned to four groups on the basis of their inclination to follow scent trails. See text for classification details.

ited by adults and concluded that neonates follow knowledgeable conspecifics short distances to the den site.

Among snakes, pheromones facilitate aggregation for both sexual and non-sexual purposes. Nonsexual aggregations probably form when snakes follow conspecifics of both sexes (Burghardt, 1980; Dundee and Miller, 1968; Graves et al., 1986; Heller and Halpern, 1981); however, during the mating period, the trailing response is clearly sex-limited (i.e., males trail females but not other males, and females do not trail either sex; Ford, 1986). Thus, it is unclear whether separate chemical cues are involved. Although further study is needed to fully characterize these substances, the pheromone permitting *C. viridis* neonates to locate dens is likely a skin lipid (Graves et al., 1986).

Snake locomotion is well suited to the deposition of a continuous scent trail (Ford, 1986), yet the hypothesis of pheromone use for den location is not without conceptual problems. One criticism is that snakes would have an even chance of following a scent trail in the wrong direction. Ford and Lowe (1984), however, cleverly demonstrated that snakes gain directionality information from the differential placement of scent on objects contacted by the trail-forming individual. Nevertheless, the first snake to arrive at a den would not have the benefit of these cues. It is probable that older, experienced snakes may relocate traditional dens using other means, e.g., solar and celestial orientational cues (Landreth, 1973; Newcomer et al., 1974), topographic landmarks (Parker and Brown, 1980), and polarized light (Lawson, 1985). Naive snakes, such as neonates and juveniles (in the case where snakes hibernate apart from adults their first winter; Gregory, 1982, 1984), may rely more heavily on pheromones for den location than do adults. This contention is consonant with the finding in the present study that nontrailing *T. sirtalis* were significantly (Kruskal-Wallis, $P = 0.02$) larger (older?) than trailing snakes; it is also supported by the relatively late arrival of smaller snakes at some communal dens (Costanzo, 1986; Gregory, 1982, 1984; Parker and Brown, 1980), and by Lawson's (1985) suggestion that solar orientational abilities are less well developed in younger *T. radix*. Since many hibernacula are inconspicuous landscape features, orientational cues alone seem insufficient to precisely locate den sites. A more parsimonious explanation is that a combination of orientational, landmark, and pheromonal cues are involved (Brown and MacLean, 1983; Gregory et al., 1987; Hirth, 1966; Lawson, 1985; Parker and Brown, 1980).

It is not surprising that snakes inhabiting high latitudes show remarkable abilities to relocate traditional dens during lengthy autumnal migrations. The original northward expansion of species' ranges doubtless depended upon the evolution of ecophysiological adaptations to colder environments and the use of winter refuges that enhanced survival. Although den fidelity typically is high, it is also imperfect (Gregory, 1984). Accordingly, individuals that located (perhaps during summer forays) and used suitable hibernacula likely prospered and

established new communal dens. The progressive northward expansion of snake populations probably was associated with increasing selection pressures for the evolution of effective mechanisms (including pheromones) for den relocation and possibly relied on traditional, communal dens as "stepping stones." This may partly explain why communal denning behavior is more prominent in snake populations near the northern extent of the species' range (Gregory, 1982, 1984). Garter snakes inhabiting milder climates (with presumably less stringent requirements for overwintering sites and consequently a greater abundance of suitable dens) show reduced inclination to trail conspecifics outside of the courtship period (Ford, 1986). The role of pheromones in den location may therefore be of greatest importance in populations inhabiting the harsh environment characteristic of northern latitudes.

Most trail-following studies are performed under idealized conditions, thus their results are only suggestive of what may occur in nature. Precipitation decreases the arrival frequency of snakes at communal dens (Costanzo, 1986; Parker and Brown, 1980), perhaps by interfering with pheromone detection. Tongue flicking, a critical element in the pheromone detection process, is slowed dramatically in *T. elegans* at low body temperatures (Stevenson et al., 1985). Preliminary work in the present investigation showed that, at body temperatures of 5 and 12°C, *T. sirtalis* were largely unresponsive to the scent trails of conspecifics. Future studies should address the influence of environmental factors (e.g., temperature, precipitation, humidity, substrate texture, wind velocity, etc.) on the trail-following ability of snakes, as well as the taxonomic and geographical limits of pheromone use for den location.

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EMPIRICAL CORRELATIONS BETWEEN ELECTROANTENNOGRAMS AND BIOASSAYS FOR *Periplaneta americana*

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Abstract—Determination of electroantennograms (EAGs) with an electroantennometer having a positive and a negative peak detection option, and with a stimulus delivery device providing local stimulation of the antenna of *P. americana*, allowed for the detection of three typical EAG patterns for a wide range of compounds tested. Some of the compounds presented at least one positive EAG peak (0.1–0.3 mV), others showed a single negative EAG peak (–1.0 to –1.1 mV), and a third group had more than one negative EAG peak (–0.2 to –0.8 mV). These EAG results correlate with behavioral assays of *P. americana*. Thus compounds having a positive EAG response act as repellents, while those having negative EAG responses act as attractants, depending on concentration. EAG patterns thus can permit prediction of behavioral responses of *P. americana*.

Key Words—Electroantennography, EAG, bioassays, *Periplaneta americana*, Orthoptera, Blattidae, electroantennogram, attractants, repellents, pheromones, mimics, chemical stimulation, American cockroach.

INTRODUCTION

The electroantennography of insects has normally been done with pheromones, mimic compounds, plant volatiles and, sometimes, repellents (Andersen et al., 1987; Gothilf and Bar-Zeev, 1972; O'Connell et al., 1986; Roelofs, 1984). For *P. americana*, these studies have been done mostly with pheromones, mimics, and general odors (Manabe and Nishino, 1986; Nishino and Takayanagi, 1979). The electroantennometers used in most of these experiments have been con-

structed to detect just a single depolarization response, i.e., a negative peak if the grounded electrode is connected to the distal part of the antenna. The devices used for obtaining the EAGs normally have considered stimulation of a very wide zone of the antenna as a whole. In these conditions the reported EAG results always have shown a single depolarization, negative in sign, for the active compounds (Gothilf and Bar-Zeev, 1972; O'Connell et al., 1986). On the other hand, bioassays for determining the activity of the compounds as attractant pheromones or mimics have been carried out with compounds presenting a negative peak under the EAG recording conditions. To the best of our knowledge, no clear results have been reported about any positive EAGs.

In this work, we present the results obtained in the electroantennography of *P. americana* with a specially constructed electroantennometer that has a simultaneous positive and negative peak detection option (Perez and Rozas, 1984) and a stimulus delivery system that has been designed to work with local stimulation of the antenna (Rozas and Perez, 1987). We also present the results of the bioassays of *P. americana* with the same compounds tested in the EAGs. It will be shown that compounds with a repellent effect on the insects in the bioassay have a positive EAG; those that behave as attractants in the bioassay have a negative EAG.

METHODS AND MATERIALS

Stimulation Chemicals. A wide structural array of available compounds was tested in this study, taking in consideration the structure of some repellents (Hagenbuch et al., 1987), some chemicals present in ventral glands of *P. americana* (Fukushima et al., 1987), common solvents, and known pheromones. All of the chemicals tested were *pro analysi* reagents; before the experiments they were further purified up to 99.0–99.5% purity as determined by GC. Periplanone B was prepared according to Schreiber and Santini (1984).

Insects. The male and female *P. americana* were maintained at 23–27°C, 45 ± 10% relative humidity, and a photoperiod of 6:18 light–dark. During the day, darkness was provided by a dark crypt inside a box, which allowed restricted light access to the crypt. Adult males of similar size and weight chosen for the EAG and bioassay experiments were separated into another box five days before the experiments and kept as before in the same environment. After use in the EAG and bioassay, they were discarded.

Electroantennography. The electroantennography of *P. americana* was done with an electroantennometer previously described (Perez and Rozas, 1984) and with a specially designed stimulus delivery device (Rozas and Perez, 1987), which basically consists of a glass tube of 3.8 mm internal diameter where the antenna is held perpendicular to it. The antenna at each extreme is attached to

a conductor that consists of a silver electrode in Ringer solution. One of the conductors is coated with a copper conductive paint for electrostatic shielding. A controlled flux of air (60 ml/min) is introduced through the tube, and the chemical stimulus is injected in the airstream through a lateral capillary injector. There is no apparent fluctuation of the chemical concentration during the stimulation. This was shown by injecting a iodine vapor puff in the airstream during a control of the setup. In this way, the angle between the injector capillary and the direction of the flux was determined to be smaller than 60°. Bigger values for that angle caused variations in the flow. This device is then placed in an electrostatically shielded box.

A filter paper strip ($2 \times 0.5 \text{ cm}^2$) was impregnated with a chemical (1–50 μg). For that, a 5- μl amount of a hexane or ether solution of the chemical was applied to the filter paper strip, and then the solvent was allowed to evaporate. More volatile chemicals as carbon disulfide, chloroform, 1,2-dichloroethane, diethyl- and triethylamine, were applied neat. The impregnated filter paper was placed in a 10-ml glass syringe. After 10 ml of air had been drawn into the syringe, the odor was mixed into the filtered airstream (60 ml/min) that was continuously blown over the antennal setup by pushing the plunger quickly (ca. 30 msec), so that air including odor (ca. 1 ml) was puffed onto the antennal preparation through the 3.8-mm-diameter glass tubing. This general procedure for presenting the stimulus has been of general use for EAG determinations (Manabe and Nishino, 1986; Nishino and Takayanagi, 1979; Roelofs, 1984). As established earlier (Roelofs, 1984), we found that slight variations in the speed at which the syringe plunger was depressed and in the amount of puffed air do not seem to be critical for the resulting response intensity. Small changes in concentration or differences in compound volatility at the dosage level used also have little effect on the relative responses to a standard compound like camphor. No changes in polarity were observed.

The antenna was removed from the insect and located in the supporting tube of 3.8 mm internal diameter. There was no variation in the response if the EAG was done either with the first or the second antenna of the insect. Reliable results were obtained during the first 20 min after removing each of the antennae. Chemical stimulation was done on the central part of the antenna where most of the chemoreceptors are located (Nishino and Takayanagi, 1979). Each antenna was stimulated a couple of times with the same chemical. An interval of 30 sec between tests was used routinely. As a control, the same procedure was performed without a test chemical. This type of control was done at the beginning and at the end of each EAG determination. Ten animals or 20 preparations were used for testing a single chemical. EAG shape pattern remained unchanged. Maximum peak values had a variation lower than 8–10%.

Bioassays. The bioassays were done in a polystyrene box (Figure 1), which

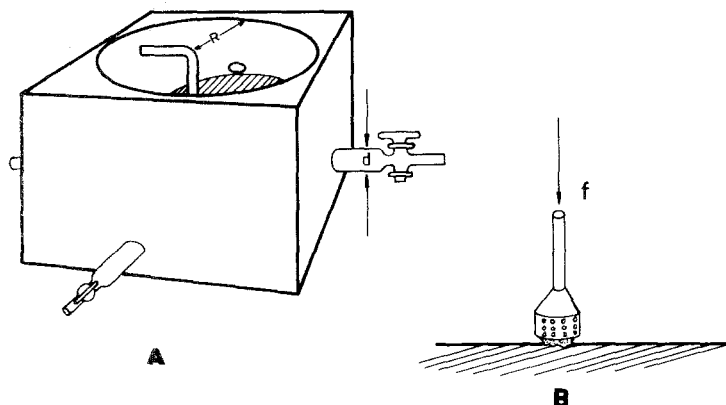


FIG. 1. (A). Box used for the bioassays of *P. americana* with different attractants and repellents; (B) amplification of the inverted grided funnel where the chemical is placed; $R = 25$ cm; vol = 157 liters; $d = 4.5$ cm; $f =$ air flow.

was kept closed during the experiments in order to simulate a habitat similar to the natural one for *P. americana*. The box was provided with four independent dark glass tubes (crypts) located on opposite faces. One adult male was located in each crypt of the box, so four adults were used in any one experiment. Each experiment was repeated twice with the same insects and then, after changing the insects, the whole cycle was repeated five times. Thus, 20 different insects were used twice for every chemical.

Between bioassays, the box was ventilated in order to start with a similar atmosphere. The chemicals to be tested, 40–55 μg , were absorbed in the usual way on a piece of filter paper. Then, the paper was located in the center of the bottom area of the box. A perforated glass funnel was inverted over the sample (see Figure 1B), through which air was swept; this air (10–20 ml/min/crypt) carried the volatiles out through a stopcock at the end of each crypt. The cover of the box was provided with a window for watching the insects every 3 min after exposing them to the chemicals. A control experiment, done without any test chemical absorbed on the piece of filter paper, revealed a random distribution of the insects in the box after a few minutes. Based on this observation, if after 20 min no insects approached to the chemical, it was considered to be a repellent (R). This behavior was checked visually through another window located at the back of each crypt. On the other hand, if the insects were all moving around the chemical during the time of observation, it was considered to be an attractant (A). These two extreme categories were used for the classification of the compounds given in Table 1.

TABLE 1. ELECTROANTENNOGRAPHY MAXIMUM PEAK VALUES AND BIOASSAY RESULTS FOR *P. americana*

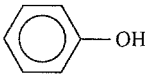
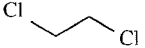
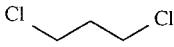

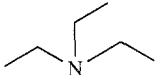
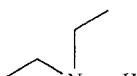
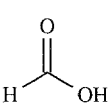
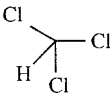
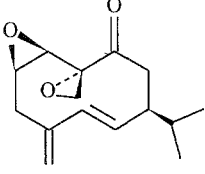
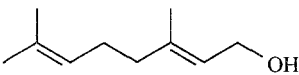
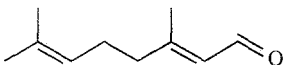
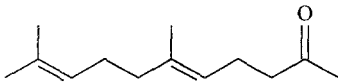
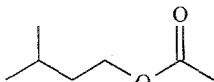
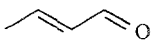
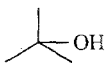
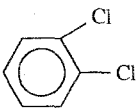
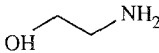
Compound	Purity (%)	EAG peaks (mV)	Bioassay
S=C=S	99.8 ^(a)	+0.30, +0.20	R
	99.5 ^(b)	+0.10, -0.20	R
	99.8 ^(a)	+0.10, -0.40	R
	99.5 ^(c)	0.10, -0.20, -0.80	R
	99.6 ^(d)	+0.10, -0.20	R
	99.4 ^(d)	+0.10, -0.60	R
	99.3 ^(d)	+0.30, -0.80	R
	99.1 ^(e)	+0.10, -0.50, -0.30	R
	99.9 ^(a)	-1.00	A
	99.6 ^(e)	-1.10	A
CH ₃ (CH ₂) ₆ CH ₂ OH	99.4 ^(e)	-0.35, -0.30	A/R
	(<i>f</i>)	-0.40, -0.38	A/R

TABLE 1. Continued

Compound	Purity (%)	EAG peaks (mV)	Bioassay
	(f)	-0.80, -0.40	A/R
	(f)	-0.36, -0.20	A/R
	99.6 ^(c)	-0.80, -0.70	A/R
	99.2 ^(c)	-0.30, -0.70	A/R
	99.5 ^(d)	-0.70, -0.50	A/R
	99.2 ^(c)	0.00	
	99.0 ^(c)	0.00	

^a Aldrich reagent with no further purification.

^b Sigma reagent purified by recrystallization.

^c Aldrich reagent purified by distillation.

^d Merck reagent purified by distillation.

^e prepared by synthesis.

^f Aldrich reagent purified by vacuum fractionated distillation up to 89.5–99.0%.

RESULTS

Typical EAGs are given in Figure 2. Table 1 gives the maximum positive or negative values of the EAG peaks for the different chemical stimuli tested. As can be seen from Table 1, the EAGs obtained by a narrow local stimulation (3.8 mm) on the central part of the antenna of *P. americana* showed three typical patterns: (1) those with a double positive response (Figure 2A) or with a positive response followed by a normal negative one, (2) those with a single negative peak as is normally observed in EAG, and (3) those with a double negative response (Figure 2B).

The bioassay results showed a very interesting tendency (Table 1): compounds with repellent effects, designated as R, presented under EAG experi-

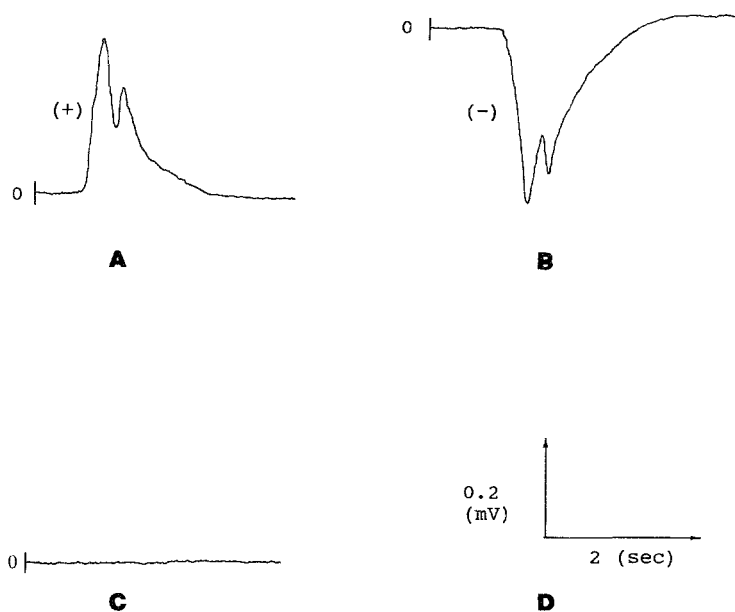


FIG. 2. Typical electroantennograms of *P. americana* to different stimuli: (A) EAG with CS₂; (B) EAG with *n*-octanol; (C) control stimulation EAG with no test chemical; (D) voltage-time scale used in the EAGs.

ments the characteristic pattern described in (1) with at least one positive peak. This was not observed for the rest of the compounds studied. On the other hand, compounds that were attractive on bioassay, designated as A in Table 1, had pattern (2) in EAG with a single negative peak. Compounds that belong to the pattern described as (3) under EAG conditions (double negative response) had a bioassay behavior that depended on concentration; these compounds were attractive at low concentrations (40–50 μg absorbed on the filter paper), but when their concentration was increased stepwise over 50 μg and in some cases up to 500 μg , they became repellent to insects; that is why they were designated as A/R compounds in Table 1. Bioassays with R or with A type chemicals showed no dependence with the concentration of chemical stimuli. However, all the EAGs and bioassays represented in Table 1 are compared in the range of 1–50 μg of chemical stimulus.

Mixtures of attractants (A) with repellents (R) that had a positive peak on the EAGs always showed a repellent effect during the bioassays. That general fact was first detected by working with 1:1 molar mixtures of A and R compounds. Other molar proportions showed a repellent effect during the bioassay as long as the EAG detected a positive peak. For mixtures between A and A/R compounds, the EAG had a double negative peak, and the final bioassay

result was attraction if the stimulus was less than 50 μg or repellency at higher concentrations of the mixture. The last compounds in Table 1 did not show bioactivity either on EAG or bioassay.

DISCUSSION

The use of an electroantennometer with positive and negative peak detection options (Perez and Rozas, 1984) and the use of a selective stimulus delivery device (Rozas and Perez, 1987) allows one to do EAG determinations by selective stimulation of particular zones of the antenna of *P. americana*. Under these conditions, very characteristic patterns for the EAGs were found. These patterns are quite reproducible, and for attractant stimuli, we have found the usual reported EAGs (Nishino and Takayanagi, 1979; Roelofs, 1984) with maximum values of -1.0 to -1.1 ± 0.08 mV for 40 determinations. Meanwhile EAG results for compounds acting as repellents are less common, and the reported ones (Gothilf and Bar-Zeev, 1972) have not revealed the existence of any positive peak, probably because of the EAG set-up used. In our hands, positive peaks obtained in the EAG of repellent compounds showed intensities varying between 0.1 and 0.3 ± 0.01 mV for 40 determinations. Polarity was kept constant for all of the repetitions.

Bioassays carried out with the same chemicals tried on EAG correlate fairly well with the EAG patterns, as can be seen in Table 1. All of the repellent compounds, as determined by bioassay, show at least one positive peak when analyzed by EAG. This fact seems to be very significant for the prediction of a general repellent behavior of a particular compound by the insect. At the moment, we do not have any reasonable explanation for these experimental EAG findings. However in terms of the affinity of a compound for the sex pheromone receptors, intrinsic activity EAG responses ranging from negative to positive signs have been theoretically expected for *P. americana* (Manabe and Nishino, 1986). On the other hand, attractant compounds behave as usual and show a single negative peak on EAG. Both of these kinds of compounds, R and A, showed a concentration-independent behavior. A/R chemicals in Table 1 presented at least two negative peaks on EAG, and their behavior on bioassay was dependent on their concentration: they are attractive at low concentrations (under 50 $\mu\text{g}/\text{filter paper}$) and become repellents at higher concentrations.

These correlations seem to us to provide striking experimental evidence of the existence of a relationship between the results that can be obtained by stimulating the insect as a whole on bioassay and parts of a removed antenna on EAG. In spite of the difficulty in understanding of these facts, the coupling of

EAG and bioassay experiments should be studied further in order to gain new insights into the correlation of the results from these two simple techniques and the structure of bioactive compounds. This type of study also can allow one to know more about the types of sensillae distributed along the antenna and individual responses.

Until now, researchers have done EAGs with nonselective stimulus delivery systems and with electroantennometers having a single peak detection capability. From these, the chemical has been considered to be or not to be a pheromone or a mimic (Manabe and Nishino, 1986). A careful EAG and bioassay could change such a deduction, as we have found for *P. americana*.

In this way, after the EAG has been carried out, it would be possible to predict if the analyzed chemical should be a repellent or an attractant to the insect according to the pattern displayed and to the empirical relationship found between EAG and bioassay experiments for *P. americana*, which could be confirmed by doing some bioassays at different concentrations.

Therefore, a detailed EAG together with bioassay experiments for insects seem highly advisable in the search for active compounds, for a better understanding of chemical communication of insects, and for their possible control.

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ECOLOGICAL ADAPTATION OF AN
ARISTOLOCHIACEAE-FEEDING SWALLOWTAIL
BUTTERFLY, *Atrophaneura alcinous*,
TO ARISTOLOCHIC ACIDS

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Abstract—Seven analogs of aristolochic acids (I, II, III, B, C, D, and E) were isolated from the leaves of *Aristolochia debilis* and characterized as the larval feeding stimulants of an Aristolochiaceae-feeding swallowtail butterfly, *Atrophaneura alcinous*. Aristolochic acids showed synergistic activity in combination with the water-soluble components in the leaf extract. Aristolochic acids were detected in the body tissues and specialized organs throughout all life stages of *A. alcinous*. Larval osmeterial fluid contained aristolochic acids as high as 2% of the secretions, the compositions of which were similar to that found in the leaf extracts. In contrast, the eggs selectively contained aristolochic acids I and II. These two acids were present both in the egg yolk and egg coating material as well as in the collateral glands (glandulae sebaceae) of the adult females. The cannibalistic activity of the larvae against the eggs and pupae seemed to be triggered by aristolochic acids present on the egg surface and pupal cuticle, suggesting a possible adaptive mechanism in this species. Aristolochic acid I deterred feeding of tree sparrows, which suggested a defensive role against vertebrate predators.

Key Words—*Atrophaneura alcinous*, Lepidoptera, Papilionidae, *Aristolochia debilis*, Aristolochiaceae, aristolochic acid, sequestration, feeding stimulant, defense, cannibalism, osmeterium, sparrow, *Passer montanus*.

INTRODUCTION

A large number of swallowtail butterfly species (Papilionidae) feed on the plant family Aristolochiaceae. The host adaptation in some of these species is associated with the host-originated toxic substances, aristolochic acid (AAs) (Rothschild et al., 1972). A Japanese pipevine swallowtail, *Atrophaneura alcinous*, feeds exclusively on *Aristolochia* plants. The adult butterfly exhibits aposematic red coloration on its body and hindwings and is supposed to be an unpalatable model for several mimicry species. *A. alcinous* appeared to sequester AAs in body tissue as in other troidine species (Euw et al., 1968; Urzúa et al., 1983; Urzúa and Priestap, 1985). Not only adults but also eggs, larvae, and pupae of *A. alcinous* have been found to store AAs in high concentration. It is here demonstrated that AAs act as deterrent allomones against birds. Chemical sequestration and consequent protection require appropriate host recognition. Adult females of *A. alcinous* have been shown to be stimulated to oviposit by AAs, in addition to other components in their host plant, *A. debilis* (Nishida and Fukami, 1989). Furthermore, AAs were found to initiate feeding activity by *A. alcinous* larvae. We describe here the chemical basis of the ecological adaptation of *A. alcinous* mediated by its host plant chemicals, the AAs.

METHODS AND MATERIALS

Instruments. Mass spectra (MS) were obtained with a Hitachi M-80 mass spectrometer at 70 eV (in-beam). Ultraviolet (UV) spectra were obtained with a Shimadzu UV-360 recording spectrophotometer, and infrared (IR) spectra were measured with a Shimadzu IR-400 spectrometer. Proton and carbon-13 nuclear magnetic resonance ($[^1\text{H}]$ NMR, $[^{13}\text{C}]$ NMR) spectra were obtained with a JEOL JNM FX-90Q spectrometer (90 MHz) in d_6 -dimethylsulfoxide using TMS as an internal standard, unless otherwise mentioned. A JEOL JNM FX-200 was also employed for the measurements of $[^1\text{H}]$ NMR spectra at 200 MHz. The letters s, d, t, q, and m represent singlet, doublet, triplet, quartet, and multiplet, respectively. Melting points (mp) are uncorrected.

Larval Feeding Stimulant Bioassay. Newly hatched first instar larvae were used for feeding tests within 24 hr after emergence. (The eggs had been removed from the host-plant leaves before hatching to prevent the larvae from direct contact with the host chemicals.) Solutions of test samples of given concentrations were applied as bands (5×10 mm), at least 15 mm apart, to a thin tissue strip (5 mm width, Kimwipe S-200, Kimberly-Clark Corporation) as shown in Figure 1. Doses were expressed either as gram-leaf equivalent (GLE) per 50-mm^2 area of the tissue strip or as the absolute amount applied to the test area. The test strip, after misting with distilled water, was placed in a Petri dish (30

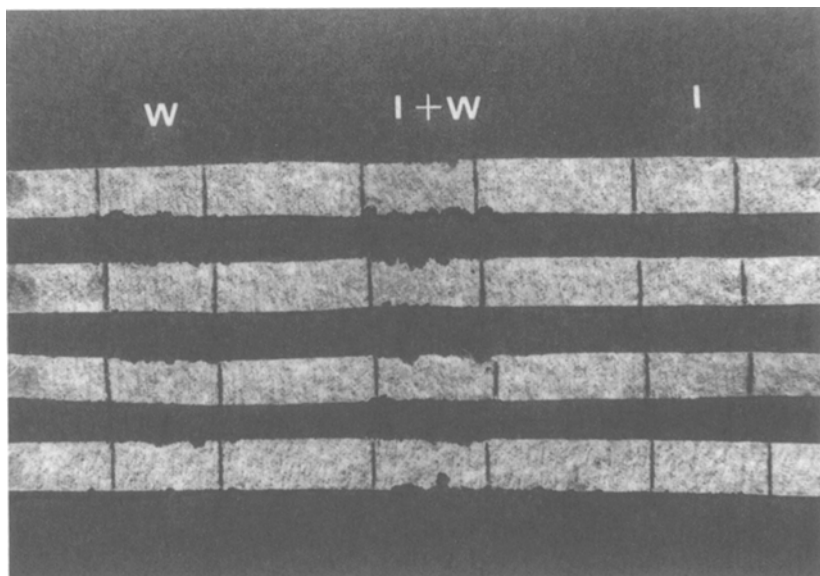


FIG. 1. Feeding responses of *Atrophaneura alcinous* larvae to the water layer of *Aristolochia debilis* (W), aristolochic acid I (I), and a mixture of both (W + I). The first-instar larvae actively bit the area where I + W was applied.

mm inner diameter), and several larvae (about two individuals per 50-mm² area) were released. After 18 hr (8:10 dark–light photoperiod, 22–24°C), the feeding activity of the insects was measured. Each 5 × 10 mm square was divided into 50 sections (each 1 × 1 mm), and the sections where the bites were observed were counted. The activity was expressed as a percentage of the bitten sections per 50 sections. Usually larvae selectively bit into the area where diluted methanolic extracts of *A. debilis* were applied (1 mGLE/50 mm²), and consumed 10–25 sections within a test period. The mean number of counts was obtained from at least eight duplicate tests.

Extraction and Fractionation of Aristolochia Leaves. The aerial parts (leaves and shoots) of *A. debilis* were collected in Kyoto prefecture in September 1984 and extracted with methanol (7 liters/kg of plant). The methanolic extract (50 g/kGLE) was dissolved in water (1 liter) and washed with ether (400 ml × 3), followed by ethyl acetate (300 ml × 3), and butanol (400 ml × 3). The combined ethereal layer (12.5 g) was extracted successively with saturated sodium bicarbonate (200 ml × 3), 1 N HCl (200 ml × 2), and saturated sodium chloride (150 ml × 4), and dried over anhydrous sodium sulfate to obtain the neutral fraction (12 g). The sodium bicarbonate layer was extracted with 20%

methyl ethyl ketone in ether (100 ml \times 3) after acidification to pH 2 with hydrochloric acid. The upper phase was washed with saturated sodium chloride and dried over anhydrous sodium sulfate. Removal of the solvent yielded a yellow solid mass of acidic components (0.30 g). The basic components (6 mg) were obtained from the hydrochloric acid layer after adjusting to pH 12 with sodium hydroxide and extracting with ether.

Isolation of AAs. The ethereal acidic fraction was composed mainly of several analogs of AAs, which were monitored by high-performance liquid chromatography (HPLC) using Radial Pak, μ Bondapak C₁₈, 100 mm \times 8 mm ID (Waters Associates), eluted with a mixture of methanol, water, and formic acid (66:33:1) at 3 ml/min (detector: Jasco UVIDEC-100-II, 254 nm). The retention times (R_t) of AAs C, B, D, II, III, E, and I were 2.7, 3.4, 3.8, 4.8, 5.3, 5.9, and 7.0 min, respectively. AA-I (80 mg) was crystallized from an acetone solution of the acidic fraction. The residual acidic mixture (0.25 g) was chromatographed on a reverse-phase column (8 g of ODS-W, microbead silica gel 5D, 100–200 mesh, Fuji-Devison Chemical Ltd.) eluted with a mixture of methanol–*N,N*-dimethylformamide–water–acetic acid (60:10:30:1). The first 40-ml portion of the eluate was rich in the phenolic AAs(B, C, and D), and the next 40-ml portion was mainly composed of AA-I, II, III, and E. Aliquots of these fractions were finely separated under the HPLC conditions mentioned above, and each isolated component was further purified by recrystallization from mixtures of benzene and acetone. The approximate yields of AAs from 1 kGLE were as follows: I = 120 mg, II = 20 mg, III = 3 mg, B = 80 mg, C = 10 mg, D = 3 mg.

AA-I. mp 280°C (decomp.). MS: m/z (%) 341(M⁺, 2), 296(2), 293(100), 278(76), 263(2), 250(24), 164(21), 137(12), 124(10), 43(26). IR: ν cm⁻¹ (KBr) 3500–2400 (broad), 1680, 1595, 1525, 1465, 1445, 1410, 1370, 1345, 1275, 1250, 1145, 1035, 995, 945, 900, 815, 755. [¹H]NMR: δ 8.50(1H, s), 8.46(1H, d, J = 8.5), 7.79(1H, s), 7.72(1H, t, J = 8.5), 7.25(1H, d, J = 8.5), 6.45(2H, s), 4.02(3H, s). [¹³C]NMR: δ (C–H coupling pattern) 167.6(s), 156.2(s), 145.8(s, 2C), 145.5(s), 131.3(d), 129.7(s), 124.3(s), 119.3(d), 118.7(s), 118.3(d), 117.2(s), 116.8(s), 112.1(d), 108.7(d), 102.8(t), 56.1(q).

AA-II. mp 268°C (decomp.). MS: m/z (%) 311(M⁺, 5), 293(3), 278(2), 263(100), 235(4), 207(6), 179(11), 177(15), 164(6), 150(26), 131(10), 99(5), 75(15), 43(10). [¹H]NMR: δ 9.02(1H, broad d, J = 8.5), 8.56(1H, s), 8.22(1H, double d, J = 8.5 and 1.7), 7.8(2H, m), 7.80(1H, s), 6.50(2H, s). [¹³C]NMR: δ 167.6, 146.1, 145.9, 145.8, 130.3, 128.7, 128.6, 128.5, 126.4, 125.7, 124.3, 117.2, 111.8, 102.9.

AA-III. mp 260–265°C (decomp.). [¹H]NMR: δ (in d₇-dimethylformamide) 8.58(1H, d, J = 2), 8.42(1H, s), 8.19(1H, d, J = 9), 7.75(1H, s), 7.45(1H, double d, J = 9 and 2), 6.47(2H, s), 4.95(3H, s).

AA-B. mp 274°C (decomp.). MS: m/z (%) 357(M⁺, 32), 309(93), 294(100), 266(26), 208(25), 181(32), 131(33), 119(30), 69(76), 42(58). UV:

λ_{\max} nm (ϵ) 263(32,600), 311(11,800). IR: ν cm^{-1} (KBr) 3480, 3400-2400 (broad), 1670, 1598, 1525, 1472, 1435, 1405, 1360, 1345, 1325, 1260, 1210, 1190, 1160, 1130, 1095, 1035, 1002, 975, 935, 915, 892, 875, 855, 828, 820, 754, 738. [^1H]NMR: δ 8.69(1H, d, $J = 9$), 8.44(1H, s), 7.72(1H, s), 7.51(1H, d, $J = 9$), 6.46(2H, s), 3.99(3H, s). [^{13}C]NMR: δ (C-H coupling pattern) 167.7(s), 148.9(s), 146.6(s), 145.8(s), 145.0(s), 142.9(s), 124.4(s), 124.1(s), 122.8(d), 121.9(s), 121.6(d), 119.4(d), 117.9(s), 115.5(s), 110.9(d), 102.7(t), 61.0(q).

AA-C. mp 272°C (decomp.). MS: m/z (%) 327(M^+ , 8), 293(8), 279(100), 193(21), 139(20), 108(12), 69(33), 55(40), 42(83). IR: ν cm^{-1} (KBr) 3470, 3400-2400 (broad), 1675, 1602, 1510, 1425, 1370, 1335, 1290, 1255, 1205, 1132, 1115, 1060, 1040, 930, 886, 870, 815, 720, 758. [^1H]NMR: δ (in d_6 -acetone + d_5 -pyridine, 200 MHz) 8.62(1H, d, $J = 2$), 8.40(1H, s), 8.04(1H, d, $J = 8.5$), 7.85(1H, s), 7.83(1H, double d, $J = 8.5$ and 2), 6.40(2H, s).

AA-D. mp 260°C (decomp.). MS: m/z (%) 357(M^+ , 0), 309(3), 294(9), 266(5), 149(7), 83(15), 43(100). [^1H]NMR: δ (in d_6 -acetone + d_5 -pyridine, 200 MHz) 8.62 (1H, s), 8.23(1H, d, $J = 2$), 7.80(1H, s), 6.90(1H, d, $J = 2$), 6.45(2H, s), 4.08(3H, s).

AA-E. MS: m/z (%) 371(M^+ , 3), 323(100), 308(29), 280(7), 278(2), 266(2), 250(5), 225(3), 141(2), 72(5). [^1H]NMR: δ 8.77(1H, d, $J = 9$), 8.45(1H, s), 7.75(1H, d, $J = 9$), 7.74(1H, s), 6.48(2H, s), 4.00(3H, s), 3.99(3H, s).

Methylation of AAs. AA-I, C, III, B, and E were individually methylated with excess diazomethane in ether.

AA-I methyl ester. mp 285°C (decomp.). MS: m/z (%) 355(M^+ , 19), 324(7), 309(100), 293(78), 279(59), 267(19), 251(23), 208(8), 151(12), 137(8), 76(4). IR: ν cm^{-1} (KBr) 2880, 1718, 1597, 1542, 1465, 1447, 1430, 1400, 1380, 1345, 1330, 1310, 1273, 1242, 1222, 1190, 1140, 1115, 1078, 1058, 1042, 962, 940, 903, 893, 872, 818, 810, 798, 786, 772, 752.

AA-C 6-O-Methyl Ether Methyl Ester. mp 273°C. IR: ν cm^{-1} (KBr) 2850, 1715, 1612, 1525, 1510, 1495, 1460, 1435, 1410, 1378, 1365, 1343, 1305, 1280, 1260, 1230, 1200, 1170, 1132, 1115, 1040, 1020, 1000, 938, 900, 870, 857, 815, 793, 777, 760, 720, 710.

AA-III Methyl Ester. The IR spectrum (KBr) was superposable on that of AA-C 6-O-methyl ether methyl ester.

AA-B 7-O-Methyl Ether Methyl Ester. mp 268°C. MS: m/z (%) 385(M^+ , 42), 353(8), 339(100), 324(24), 309(35), 296(15), 281(13), 266(30), 253(8). IR: ν cm^{-1} (KBr) 2900, 1710, 1690, 1520, 1475, 1430, 1380, 1340, 1320, 1290, 1265, 1245, 1215, 1195, 1170, 1140, 1100, 1080, 1050, 1030, 1000, 980, 930, 910, 880, 855, 815, 790, 780, 745.

AA-E Methyl Ester. The MS and IR spectra were identical to those of AA-B 7-O-methyl ether methyl ester.

HPLC Analysis of AAs in A. alcinous Tissues at Various Stages. The con-

tents of AA analogs in the larval osmeterial fluid, pupae, adults, and eggs were quantified by means of HPLC using a reverse-phase column (Capcell Pak C₁₈ S-5 μm , 250 mm \times 4.6 mm ID eluting with 60% methanol plus 1% acetic acid in water (1 ml/min) as shown in Figure 3. The chromatograms were monitored by UV detection at 254 nm (R_t : AA-C = 4.9, B = 6.0, D = 6.3, II = 10.1, III = 10.9, E = 11.1, I = 13.5 min). All the insects used here were reared in outdoor screen cages, and fed with fresh leaves of *A. debilis* during their larval stage. As a general procedure for preparation of AA fractions, the insect bodies or the organs were homogenized and extracted with various solvent mixtures. Acetone was used preferably for extraction because of the high solubility of AAs in the solvent, but it was usually mixed with other solvents (ethanol, methyl ethyl ketone, and benzene). The crude extracts were subjected to acid-base extraction using ether, saturated sodium bicarbonate, and 1 N hydrochloric acid in the same manner as described above for fractionation of *A. debilis* leaves. The ether-soluble acidic fractions were passed individually through a Sep-pak C₁₈ cartridge (Waters Associates), and the aliquots were subjected to HPLC analysis.

Larval osmeterial fluid was collected from the fifth-instar larvae by using glass capillary tubes while the eversible glands were extruded (Figure 2A). The glandulae sebaceae were dissected out from the female abdomen (100 females 7–12 days old, extracted with an ethanol-acetone mixture (1:1, 20 ml \times 2), Figure 2C). In order to obtain the egg yolk, full grown eggs were taken out from the female's body (5–10 days old, Figure 2B and C), crushed in an ethanol-acetone mixture (100 μl /egg), and then extracted. Egg coating was obtained by wiping the resin-like material on the egg shell (Figure 2B) immediately after the eggs were deposited on a piece of filter paper (Nishida and Fukami, 1989). Pupal rinse was obtained by washing the pupae (at least 10 days after pupal ecdysis) in a mixture of acetone and hexane (1:1, 2 ml/pupa) for 15 sec.

Deterrence Bioassay of AAs against Sparrows. Two square plates (16 \times 16 cm) were placed next to each other in an outdoor bird-feeding arena (Kyoto, May 1987). Thirty rice grains treated with an acetone solution of AA-I were placed in one plate, and 30 untreated grains (solvent blank) were placed in the other plate. The number of grains from the initial 30 grains were counted after exposure to sparrow feeding (Nishida et al., 1988).

RESULTS

Larval Feeding Stimulants. Methanolic extracts of *A. debilis* stimulated feeding by *A. alcinous* larvae when the solution was applied to a piece of thin tissue. In particular, the first-instar larvae, which had no experience of direct contact with the host plant leaves, were strongly arrested and bit into the tissue

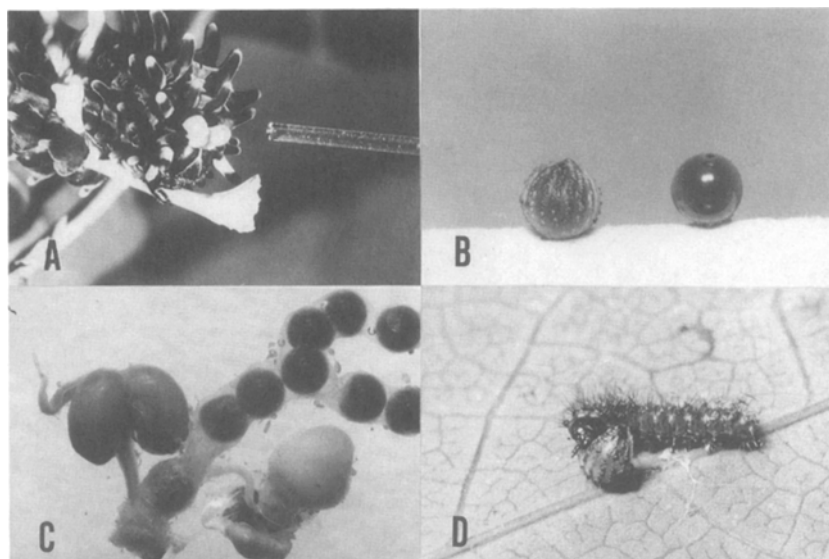


FIG. 2. (A) An *Atrophaneura alcinous* larva everting its osmeterium when the posterior end of the larva was squeezed. The osmeterial fluid can be introduced in a glass capillary tube. (B) Eggs of *A. alcinous* normally oviposited (left), and dissected from oviduct. (C) Ovarian view of a mature adult of *A. alcinous*. The glandulae sebaceae (left) is connected to the near end of the oviduct (bottom). (D) Egg cannibalism by a newly hatched first-instar larva. The larva consumed one whole egg within an hour and doubled in weight.

fiber where the extracts had been applied. In order to examine the feeding-stimulant activity of the host plant extract, thin tissue strips (Kimwipe, 5 mm in width) were used as a biting substrate (see Figure 1). A methanolic extract of *A. debilis* (aboveground parts) was fractionated into ether, ethyl acetate, butanol, and water layers by solvent extraction. Table 1 test series 1 shows the mean biting counts of each fraction at a dose of 1 mGLE/50 mm². The water layer was found to be the only fraction that exhibited the activity, but it appeared to be weak compared with that of the methanolic extract. However, the activity of the water layer was significantly increased up to the level of the original methanolic extracts by adding the inactive ether layer. The synergistic ether layer was then separated into acidic, basic, and neutral fractions, in which only the acidic fraction was found to be responsible for inducing the significant feeding activity in combination with the diluted water layer (Table 1 test series 2).

The acidic layer consisted mainly of several analogs of AAs (I, II, III, B, C, D, and E). Each AA analog was isolated by combination of preparative HPLC and crystallization. Figure 3 (top) shows a typical HPLC profile of AAs

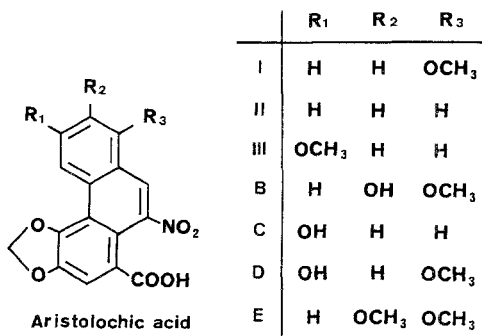
TABLE 1. FEEDING-STIMULANT ACTIVITY OF *Aristolochia debilis* FRACTIONS AND ARISTOLOCHIC ACIDS (AAs) IN LARVAL FEEDING BIOASSAY

Test series ^a	Sample	Biting counts (Mean ± SD)
1	Blank	0.3 ± 0.5
	Methanolic extract	17.8 ± 2.2
	Ether layer	0.5 ± 0.7
	EtOAc + BuOH layer	0.5 ± 0.9
	Water layer (W)	8.3 ± 1.7
	Ether layer + W	16.2 ± 3.5
2	Blank	0.7 ± 1.0
	W	2.7 ± 2.1
	W + Ether layer	15.0 ± 3.0
	W + Neutral fraction	4.7 ± 4.6
	W + Basic fraction	3.9 ± 2.6
	W + Acidic fraction	17.4 ± 2.8
3	Blank	0.8 ± 0.9
	W	3.0 ± 2.5
	W + AA-I	15.6 ± 4.1
	W + AA-II	8.9 ± 3.6
	W + AA-B	8.3 ± 4.2
	W + AA-C	7.5 ± 3.7

^aDose: 1 mGLE/50 mm² in test series 1; 0.3 mGLE (W) and 1 mGLE (each fraction)/50 mm² in series 2; and 0.3 mGLE (W) and 0.3 μg (AAs)/50 mm² in series 3.

in the young leaf extract. Each AA analog was individually tested at a 0.3-μg dose in combination with the water layer (Table 1 test series 3). AA-I exhibited distinct synergistic activity. The activities of AA-II, B, and C seemed to be relatively weak. Preliminary experiments also indicated that AA-III, D, and E also act as synergists to some extent on the water layer.

Identification of AAs. Several AA analogs have been previously isolated from *A. debilis* (Scheme 1). Tomita and Kura (1957, 1959) isolated AA-I(A), B, and C from the root. The authentic specimens of AA-B and C, kindly provided by Professor Tomita, coincided in all respects with our samples of B and C. Although AA-C was determined as 3,4-methylenedioxy-6-hydroxy-10-nitrophenanthrene-1-carboxylic acid (Sasagawa, 1962; Kupchan et al., 1965), the structure of AA-B has been uncertain (Tomita and Sasagawa, 1959). As shown in the physical data section, spectrometric data of AA-B assigned its structure to be 3,4-methylenedioxy-7-hydroxy-8-methoxy-10-nitrophenanthrene-1-carboxylic acid. The position of the 8-methoxy group was unequivocally determined by measurements of a nuclear Overhauser effect between the neighboring protons (12% increase of H-9 (δ 8.44) by an irradiation of the methoxy proton



SCHEME 1.

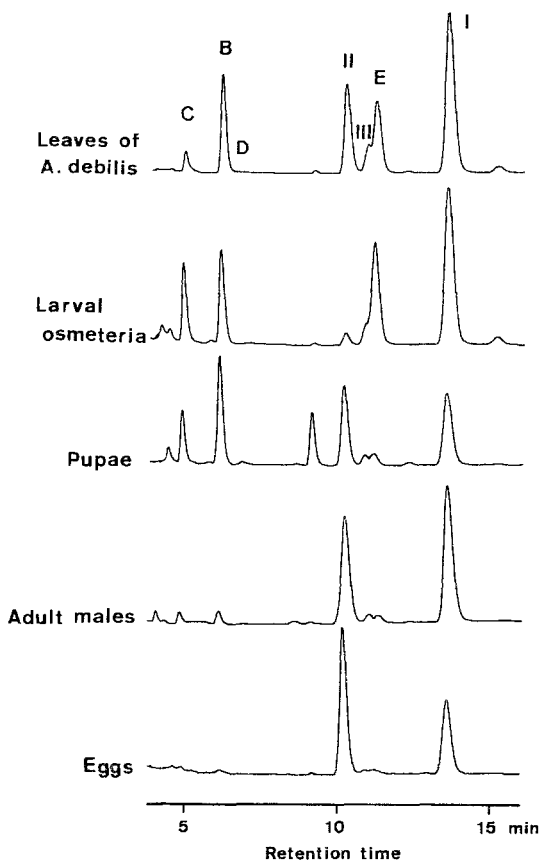


FIG. 3. Liquid chromatograms of aristolochic acids in the fresh leaves of *Aristolochia debilis*, larval osmeterial secretion, pupal whole body, adult male body, and whole eggs. The chromatogram was done on a Capcell Pak C₁₈ (S-5 μm, 250 × 4.6 mm ID), eluting with 60% methanol + 1% acetic acid in water at a flow of 1 ml/min, monitored by UV detector (254 nm).

at δ 3.99). Because AA-E (molecular weight = 371) gave the same methylated product as that of AA-B when reacted with diazomethane, AA-E was verified as a 7,8-dimethoxy analog of AA. Chen et al. (1981), however, already reported these two AA analogs from *A. debilis* roots by the names of 7-hydroxyaristolochic acid-A and 7-methoxyaristolochic acid-A. The former compound is a synonym of aristolochic acid B. To adopt the traditional nomenclature, we here propose the name aristolochic acid E for the latter compound. AA-I, II, III, and AA-D were identified by spectrometric analysis and comparison of the physical data with those reported in the literature (Pailer et al., 1956, 1965; Pailer and Schlepplik, 1957; Pailer and Bergthaller, 1966; Kupchan and Merianos, 1968).

Sequestration of AAs at Various Stages of A. alcinous Larvae. When the larvae of *A. alcinous* are irritated, they secrete orange-colored fluid from their osmeteria, which are situated between the head and prothorax, and quickly smear the fluid onto the object (Figure 2A). The fluid possesses a characteristic odor and bitter taste. The last-instar larvae usually secrete 1–2 μ l of the fluid, which can be readily introduced into a glass capillary tube. Although the fluid is usually a clear solution, a yellow crystalline mass precipitated when the fluid was dissolved in a small quantity of 1% acetic acid in ethanol. The crystals were found to be pure AA-I (mp 280°C, decomp.). A liquid chromatogram of AAs in the intact osmeterial fluid is shown in Figure 3, in which all AA-analogs were found, exhibiting a similar profile to that of the host plant extracts. The content of the major analog, AA-I, in the osmeterial fluid of the last-instar larva was found in a range of 5–10 μ g/ μ l (Table 2). The concentration of total AAs was as high as 2% of the secretion, although the composition appeared to be variable, probably due to fluctuation of the AA composition in the host plant. Besides AAs, the osmeterial fluid contained glycerol (80 μ g/ μ l), myoinositol (10 μ g/ μ l), inosine (25 μ g/ μ l), adenosine (5 μ g/ μ l), and guanosine (2 μ g/ μ l).

Pupae. The composition of AAs in a whole body extract of *A. alcinous* pupae is shown in Figure 3. All seven AA analogs were present in the extract. The analysis of a hexane–acetone rinse of pupae revealed that a portion of the AAs was deposited on the cuticular surface. The cuticular rinse seemed to contain relatively higher quantities of minor AA analogs (III and E) than were found in the body tissue (Table 2).

Adults. Male and female butterflies were analyzed separately. The HPLC profiles were not much different between the sexes, in which AA-I and II were found in almost equal quantities (Figure 3). The content of total AAs in one female was found in a range of 100–180 μ g, and that in one male was 50–120 μ g (Table 2). The quantities of AAs in the adults seemed to be proportional to their body weight.

Eggs. Eggs of *A. alcinous* are onion shaped with a rough coating of resin-like material (Figure 2B, left). However, the eggs found in the oviducts are round and lack coating material (Figure 2B, right, and 2C). A bright orange-colored material contained in the reservoir organ (glandulae sebaceae) seems to

TABLE 2. ARISTOLOCHIC ACID (AA) CONTENTS OF *Atrophaneura alcinous* TISSUES AT VARIOUS LIFE STAGES

Sample (units)	Aristolochic acids (μg)						
	I	II	III	B	C	D	E
Larva							
Osmeteral secretion (1 μl)	8.7	0.6	1.0	4.4	1.4	0.2	5.7
Pupa							
Whole body (1 pupa)	34	35	1	40	8		1
Cuticular rinse (1 pupa)	0.5	0.6	0.5	0.5	0.1		0.4
Adult							
Male whole body (1 σ)	55	43	4	5	2		3
Female whole body (1 ♀)	78	62	5	7	12		5
Glandulae sebaceae (1 gland)	10	10	0.5	0.5	0.9		0.5
Egg							
Egg yolk (100 eggs)	13	23	0.3				0.4
Egg coating (100 eggs)	3	4					
Host plant leaf							
<i>Aristolochia debilis</i> (1 g)	320	180	25	160	15	5	170

be squeezed out during egg deposition to coat the egg surface, gluing it to the leaf. The egg coating can be readily scraped off while fresh, but it gradually becomes hard within a few hours. Thin-layer chromatography revealed that the chemical composition of the egg coating was identical to that of the viscous material contained in the reservoir organ, both being composed mainly of unidentified polar lipids. As shown in Table 2, acidic fractions of both egg coating and the glands contain AA-I and II almost in equal quantities but the other analogs were much less abundant or absent. Similarly, egg yolk also contained high concentrations of AA-I and II. A liquid chromatogram of the whole egg is shown in Figure 3.

Deterrent Effect of AAs to Sparrows. Deterrent effects of AAs were examined by the rice-grain feeding test. The rice grains treated with AA-I strongly deterred feeding of the tree sparrow, *Passer montanus*. The average number of grains remaining from an initial sample of 30 grains was 16.6 ± 10.9 at a dose of $3 \mu\text{g}/\text{grain}$, while the control grains were all consumed. AA-B also exhibited moderate deterrence to sparrows.

DISCUSSION

Aristolochic acids are secondary metabolites contained characteristically in the plant genus *Aristolochia*. Nine AA analogs, namely I(A), Ia, II, III, IV, B, C(IIIa), D(IVa) and E, have heretofore been known from plants. All contain

a 3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid structure with different functional groups at positions C-6, C-7, and C-8 (Mix et al., 1983). Among these, seven AA analogs have been verified here from the leaves of *A. debilis* as the larval feeding stimulants for a Japanese pipevine swallowtail butterfly, *A. alcinous*. AAs did not induce the phagostimulant activity by themselves, but acted synergistically in combination with unidentified component(s) in the aqueous layer. AAs have also been characterized as synergistic factors for oviposition stimulants of *A. alcinous* (Nishida and Fukami, 1989). Considering the rare distribution of AAs in the plant kingdom, it would be an efficient cue, although both stimulants are composed of multiple components. The coincidence of oviposition and larval feeding stimulants has been found in the case of pierid butterflies in which glucosinolates act as the specific stimulants (Verschaffelt, 1911; David and Gardiner, 1962).

AAs have been recognized as factors responsible for toxicity and other biological activities shown by *Aristolochia* plants (Kupchan and Wormser, 1965; Kupchan and Merianos, 1968; Moretti et al., 1979). These plants seem to have developed the rare nitrophenanthrene skeleton as chemical barriers against herbivores (Miller and Feeny, 1983). *A. alcinous* not only uses them as a host-finding cue, but also utilizes the analogs as defense substances throughout all stages of its life.

Sequestration of AAs in the adult bodies has been demonstrated among several troidine species, namely *Pachliopta aristolochiae* (Euw et al., 1968), *Battus archidamas* (Urzúa et al., 1983), and *B. polydamas* (Urzúa and Priestap, 1985), and also in an *Aristolochia*-feeding Parnassiinae, *Zerynthia polyxena* (Rothschild et al., 1972). The composition of AA analogs in each insect species varied, probably depending on the variation of AA contents in their host plants, and differences of the physiological properties in the process of sequestration. Although six AA analogs were detected from both male and female adults of *A. alcinous*, the total AA content was much higher in the female specimens, in which concentration seemed to be more or less dependent on body weight. These *Aristolochia*-feeders exhibit aposematic coloration, which suggests unpalatability against predators (Brower, 1984). In addition, the occurrence of mimic species strongly implies chemical protection afforded by AAs. Feeding deterrence of AAs to sparrows clearly suggests its defensive role against vertebrate predators. AAs seemed to play a role as a gustatory cue, but further effects of AAs as noxious emetic or toxic agents against natural enemies remain to be clarified. A preliminary feeding bioassay, however, indicated that crude extracts of butterflies and osmeterial secretion induced much stronger deterrence against sparrows, suggesting the presence of an additional chemical factor(s) incorporated besides AAs. Male adults of *A. alcinous* emit a distinguishable floral odor which is composed of a mixture of phenylacetaldehyde, linalool, and other minor

volatile components (Honda, 1980a). The characteristic odor may function as an olfactory aposematic cue in addition to its visual aposematism.

Larvae of *A. alcinous* sequester extraordinarily high concentrations of AAs in the osmeteria, exhibiting HPLC profiles similar to those of fresh leaf extracts of *A. debilis*. The concentration of total AAs in the fluid was approximately 2%. In addition, the osmeterial fluid contained 8% glycerol, 3% nucleosides (inosine, adenosine and guanosine), and 1% myoinositol as the aqueous components. In contrast to irritant fatty acid components commonly found in the last-instar larvae of *Papilio*, the osmeterial secretion of *A. alcinous* larvae contains sesquiterpene volatiles (Honda, 1980b). The extremely bitter taste of the secretion in this species seems to play an initial defensive role against predators. Although the osmeterial fluid contains such a variety of ingredients, the biological functions of each individual component remain to be determined.

The pupae of *A. alcinous* exhibit seasonal color dimorphism. The diapausing pupae are mostly grayish brown, matching the background color, and nondiapausing summer pupae are aposematic bright yellow with some red spots on the back. Deposition of AAs on the cuticular surface may be effective only by contact toward certain predatory and/or parasitic attacks. Obnoxious smell and noise production of the pupae may also account for the warning signs.

Eggs of *A. alcinous* are an aposematic reddish orange color with a rough coating of resin-like material. The chloroform-soluble fraction of the coating contained unidentified viscous lipids as the major components. A mixture of AA-I and II was found both in the egg coating and yolk. No other AAs were found in significant quantity. This may be due to the difference in the stability of AAs in the body tissue during storage. The egg coating is stored in an extraordinarily large quantity in the mother's reservoir organ, which seems to be characteristically well developed among many troidine species. A large portion of AAs in the female butterflies is thus concentrated in the reservoir and transferred efficiently to the next generation. Similarly, mustard glucosides are known to occur in the eggs of pierid butterflies, and cardiac glycosides in danaid eggs (Brower, 1984). The eggs of *A. alcinous* are often laid as a cluster. This may create a more effective warning signal to certain enemies than that of solitary eggs.

The larvae of *A. alcinous* often display cannibalistic behaviors toward eggs and pupae. Deposits of AAs on egg and pupal surfaces seem to trigger cannibalistic activity. A newly hatched first-instar larva quickly gains weight by feeding on neighboring eggs (Figure 2D). Similar behavior also occurs in other *Aristolochia*-feeding swallowtails (Young, 1977; Brown et al., 1980), danuids (Brower, 1961), and a *Heliconius* butterfly (Williams and Gilbert, 1981). Population control by cannibalism at high density might be of ecological significance since low predation due to their unpalatability sometimes results in

catastrophic outbreaks of these "poisonous" species. Although the specific kairomonal and allomonal activities discussed above may not be determined solely by AAs at each step, the presence of AAs seems to have opened up an ecological niche for *A. alcinous*.

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OVIPOSITION STIMULANTS OF AN
ARISTOLOCHIACEAE-FEEDING SWALLOWTAIL
BUTTERFLY, *Atrophaneura alcinous*

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Abstract—Oviposition stimulants of an Aristolochiaceae-feeding swallowtail butterfly, *Atrophaneura alcinous*, were isolated from the leaves of *Aristolochia debilis* and characterized as a mixture of aristolochic acids and sequoyitol. An artificial blend of these components applied to filter paper induced a significant oviposition response by the female butterflies, identical to that elicited by intact leaves of the *Aristolochia* host plant.

Key Words—Oviposition stimulant, host selection, butterfly, Lepidoptera, Papilionidae, *Atrophaneura alcinous*, *Aristolochia debilis*, aristolochic acid, sequoyitol, synergist.

INTRODUCTION

A Japanese pipevine swallowtail butterfly, *Atrophaneura alcinous* Klug (Papilionidae) feeds exclusively on the plant family Aristolochiaceae. The females lay eggs very accurately on the *Aristolochia* vines. Nishida (1977) demonstrated that methanolic extracts of the host plant, *Aristolochia debilis* Sieb. et Zucc., strongly induce oviposition by *A. alcinous* females (Figure 1). The oviposition stimulant activity of the extract appeared to be highly specific in the genus *Aristolochia*, and the female butterflies detect the essential component(s) by contact receptors located on the fifth segments of the foretarsi (Nishida, 1977). Although visual and olfactory cues are also involved at the initial phase of oviposition behavior of papilionid butterflies (Vaidya, 1969; Saxena and Goyal, 1978; Rausher, 1978), nonvolatile contact chemical stimuli play a decisive role

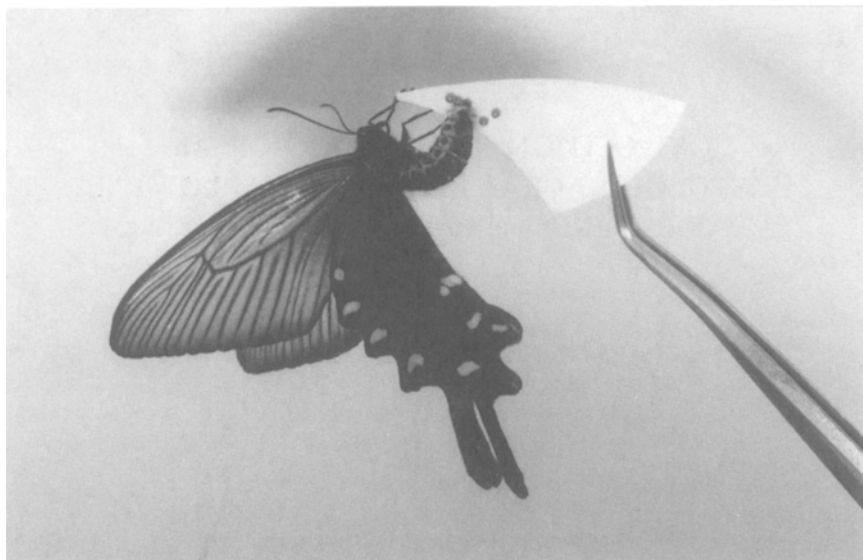


FIG. 1. Oviposition response of an *Atrophaneura alcinous* female to a piece of filter paper treated with the methanolic extract of *Aristolochia debilis*. The female curled her abdomen and laid eggs immediately after touching the filter paper with the tarsal chemoreceptors located on her forelegs.

after landing on the host-plant leaves (Nishida, 1977; Ichinosé and Honda, 1978; Feeny et al., 1983), and the essential components responsible for the oviposition have been characterized for two Rutaceae-feeding swallowtail species, *Papilio xuthus* (Ohsugi et al., 1985, Nishida et al., 1987) and *P. protenor* (Honda, 1986). A large number of papilionids are associated with Aristolochiaceae as well as Rutaceae. Thus, it is of great interest to compare the profiles of oviposition stimulants among these two major oligophagous groups, namely Aristolochiaceae feeders and Rutaceae feeders. Here we describe the isolation and characterization of the oviposition stimulants of *A. alcinous* contained in the leaves of *A. debilis*.

METHODS AND MATERIALS

Insects. *A. alcinous* at various stages were collected in Kyoto and Mie prefectures and reared for several generations on *A. debilis* vines mostly under a short photoperiod (10:14 light-dark, 22–26°C). The diapausing pupae were stored in a refrigerator (0–7°C) until use. The butterflies after adult eclosion at around 24°C were hand-paired within two days. The mated females were indi-

vidually confined in small screen cages (16:8 light-dark photoperiod) and fed a sucrose solution (5–10%) daily.

Oviposition Bioassay. The bioassay chamber (50 × 50 × 50 cm) was open in front and its interior was illuminated by a fluorescent lamp (15 W) placed behind a screen at the rear. Test samples were applied to a piece of filter paper (approximately 10 cm²) by cutting a round filter paper (9 cm diameter) into six equal fan-shaped pieces. Aliquots of 100 μl of each concentration of the sample were applied to the filter paper and allowed to dry. Doses were expressed either as gram leaf equivalents per 10 cm² of filter paper (GLE/FP) or as the absolute amount applied to the filter paper (μg/FP). Test filter papers were misted with distilled water immediately before the bioassay. Each female butterfly (3–10 days old) was placed in turn on the filter paper and kept in contact with it as much as possible during 30 sec (Figure 1). Females usually responded immediately to the standard methanolic extract of *A. debilis* (0.03 GLE/FP), curling their abdomens, and eventually laying eggs. Any females either giving a positive response to a moistened blank filter paper or giving no response to the standard methanolic extract were rejected from the test. The activity was evaluated by the percentage of females that gave a positive response at given doses. The bioassay was conducted during daylight hours.

Extraction and Fractionation. Leaves and stems of *A. debilis* (1.8 kg) were collected in Kyoto Prefecture during July 1983 and extracted with methanol (12 liters). Evaporation of the extract gave a viscous oil (83 g). An aliquot of the methanolic extract (23 g, 500 GLE) was dissolved in water (500 ml) and washed with ether (300 ml × 3), followed by ethyl acetate (150 ml × 3), and butanol (200 ml × 3) as shown in Figure 2. The yields of the ether, ethyl acetate, butanol, and water layers were 6.20, 0.20, 1.51, and 19.47 g, respectively. The ether layer was fractionated into acidic (0.20 g), basic (0.02 g), and neutral (5.79 g) fractions by using saturated sodium bicarbonate and 2 N hydrochloric acid. The water layer (2 g, 50 GLE) was dissolved in 2% acetic acid in water (5 ml) and chromatographed on a reverse-phase column (20 g of ODS-W, microbead silica gel 5D, 100–200 mesh, Fuji-Davison Chemical Ltd., packed in water, 120 mm × 20 mm ID) eluted in sequence with 100 ml of 1% acetic acid in water (W-1), 1% acetic acid + 10% methanol in water (W-2), 1% acetic acid + 40% methanol in water (W-3), and 1% acetic acid in methanol (W-4). The yield of W-1 was 1.64 g.

Instruments. Mass spectra were obtained with a Hitachi M-80 mass spectrometer. The infrared (IR) spectrum was recorded with a Shimadzu IR-400 spectrometer. [¹H]- and [¹³C]NMR spectra were obtained with a JEOL JNM FX-90Q spectrometer (90 MHz) using either DSS (for D₂O solution) or TMS (for CDCl₃ solution) as the internal standards.

Isolation of Aristolochic Acids (AAs). Four analogs of AAs (I, II, B, and C) were isolated from the ether soluble acidic fraction by means of high-per-

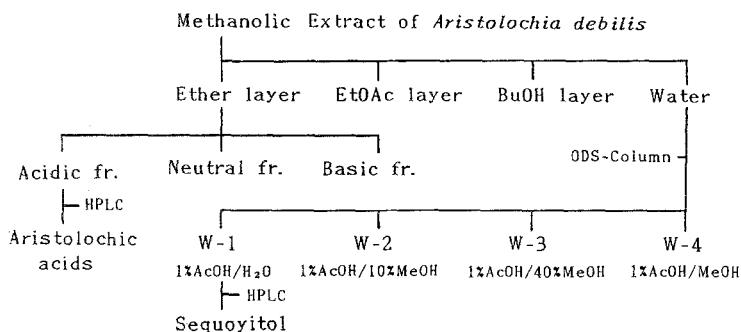


FIG. 2. Procedure for separation of the methanolic extract of *Aristolochia debilis*.

formance liquid chromatography (HPLC) using a reverse-phase column (μ Bondapak, 10 μ m, Waters Associates, 100 mm \times 8 mm ID, eluting with a mixture of methanol–formic acid–water = 66:1:33, at 3 ml/min). The retention times (R_t), melting points (mp; decomposed points) and yields (per 1 GLE) of AAs are as follows. AA-I: R_t = 7.0, mp = 280°C, yield = 120 μ g. AA-II: R_t = 4.8 min, mp = 268°C, yield = 40 μ g. AA-B: R_t = 3.4 min, mp = 274°C, yield = 80 μ g. AA-C: R_t = 2.7 min, mp = 272°C, yield = 10 μ g (Nishida and Fukami, 1989).

Isolation of Sequoyitol. Fraction W-1 was separated into 11 fractions on an HPLC column (YMC-Pack PA-24, 300 mm \times 8 mm ID, 67% acetonitrile in water, 3 ml/min) as shown in Figure 3. Fraction 5 was found to contain an active principle, sequoyitol, in high purity. In order to obtain a larger quantity of sequoyitol, the water layer (100 GLE) was passed successively through a column of cation-exchange resin (Amberlite CG-50, type 1, 160 mm \times 20 mm ID) and anion-exchange resin (Dowex-1 \times 8, 200–800 mesh, conditioned with formic acid, 150 mm \times 30 mm ID), both eluted with 500 ml of distilled water. The nonretained (neutral) fraction (1.77 g) was then chromatographed on a column of active charcoal (30 g, 160 mm \times 32 mm ID, Wako Pure Chemical Industries Ltd.) eluted with water. After elution of 300 ml of water, the remaining 100 ml eluate (42 mg) was crystallized from 70% methanol in water to yield colorless prisms of sequoyitol (yield 23 mg, mp 239–240°C).

Analysis. Found: C, 43.03%; H, 7.43%; O, 50.00%. Calcd. for $C_7H_{14}O_6$: C, 43.30%; H, 7.27%; O, 49.44%. IR: ν (nujol) 1263, 1185, 1130, 1105, 1070, 1055, 1025, 940, 930, 885. 1H NMR: δ (D_2O) 3.99(1H, triplet, J = 2.5 Hz), 3.66(2H, triplet, J = 9 Hz), 3.54(3H, singlet), 3.26(2H, double doublet, J = 2.2 and 9 Hz), 3.30(1H, triplet, J = 9 Hz). ^{13}C NMR: δ (D_2O) 86.54(C-5), 74.41(C-2), 74.11(2 \times C), 73.60(2 \times C), 61.89(OCH₃).

Sequoyitol Pentaacetate. Sequoyitol (13 mg) isolated from *A. debilis* was acetylated in a mixture of acetic anhydride (1.5 ml) and pyridine (1.5 ml) to

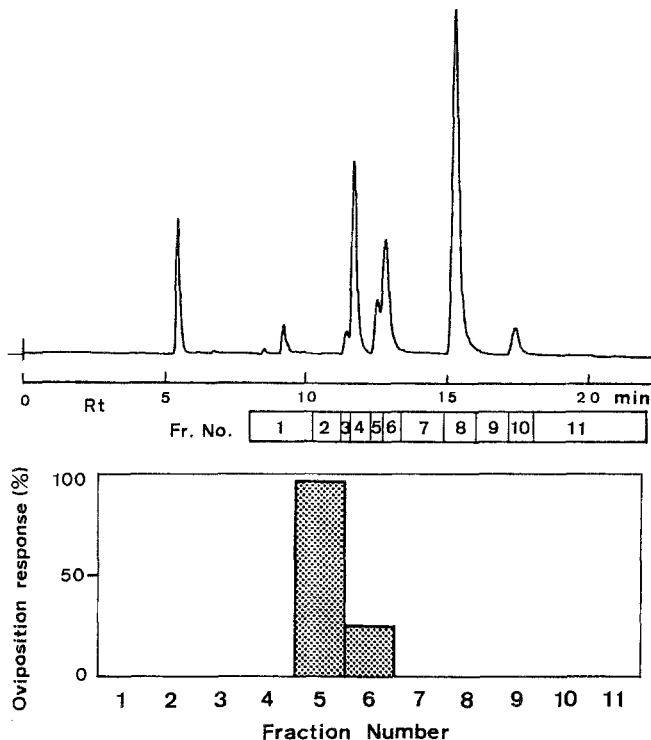


FIG. 3. Upper: Liquid chromatogram of the active fraction W-1 (YMC-Pak PA-24, 300 mm \times 8 mm ID, 67% acetonitrile in water, 3 ml/min, refractive index detection). Lower: Oviposition response of the fractions to *Atrophaneura alcinous*. Each fraction was tested at a dose of 0.03 GLE/FP mixed with 3 μ g of aristolochic acid I.

yield a pentaacetyl derivative (yield 16 mg, mp 197–198°C). MS (20eV, in-beam) m/z 404(M^+ , 0.2%), 361(2%), 346(2%), 332(5%), 301(3%), 243(20%), 182(61%), 140(77%), 43(100%). [1H]NMR: δ ($CDCl_3$) 5.56(1H, triplet, $J = 3$ Hz), 5.46(2H, double doublet, $J = 9.5$ and 10 Hz), 5.05(2H, double doublet, $J = 10$ and 3 Hz), 3.45(3H, singlet), 3.43(1H, triplet, $J = 9.5$ Hz), 2.19(3H, singlet), 2.08(6H, singlet), 1.99(6H, singlet). [^{13}C]NMR: δ ($CDCl_3$) 169.81, 169.59, 169.38, 80.12, 70.70, 68.88, 68.45, 59.94, 20.64, 20.37.

RESULTS

The components responsible for stimulating oviposition of *A. alcinous* were efficiently extracted with methanol from its host plant, *A. debilis*. As shown in Figure 4, the oviposition stimulant activity of the methanolic extract was dosage

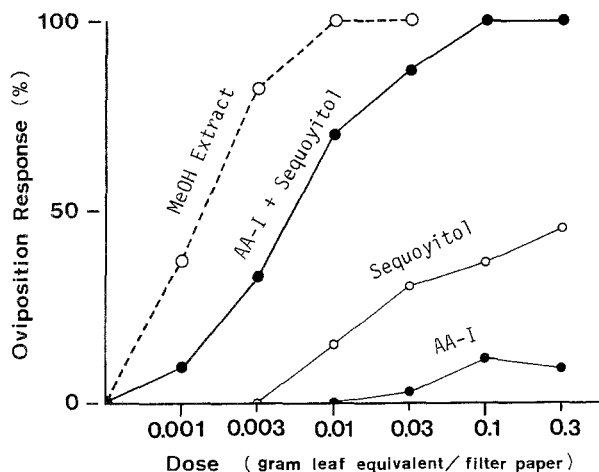
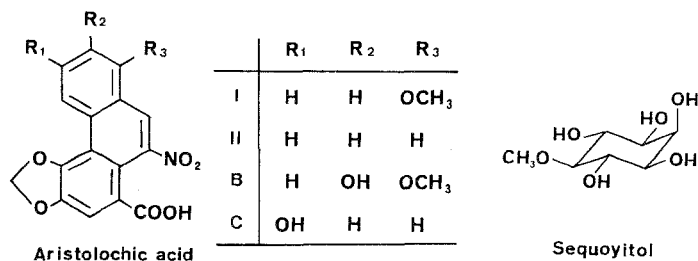


FIG. 4. Dose oviposition responses of the methanolic extract of *Aristolochia debilis*, aristolochic acid I(AA-I), sequoyitol, and a mixture of the two.

dependent, and 100% positive responses were obtained at doses of 0.01 GLE/FP and higher. The methanolic extract was separated into ether, ethyl acetate, butanol, and water layers by solvent extraction (Figure 2). The water layer was found to be the only fraction that showed significant oviposition activity by itself, but the activity appeared to be considerably lower than that of the original methanolic extract (Table 1). The original activity of the extract, however, was almost fully recovered when the ether layer was added in an equivalent dosage (0.01 GLE/FP) to the water layer. The synergistic factors contained in the ether layer were acidic in nature (Figure 2 and Table 1). The acidic fraction was found to consist mainly of several analogs of aristolochic acids (AAs). Seven AA analogs, namely I, II, III, B, C, D, and E, have been identified from *A. debilis* (Tomita and Sasagawa, 1959; Chen et al., 1981; Nishida and Fukami, 1989). All of the AA analogs bear a nitro, a carboxylic acid, and a methylenedioxy group on the phenanthrene ring, but the functional groups on the upper ring differ from one structure to another. The two major analogs, AA-I and AA-B, were found to act as synergists of oviposition activity, when combined with the water layer. As shown in Table 2, both AAs were inactive alone, but a remarkable enhancement of the activity was observed when either AA-I or AA-B was added to the water layer at a threshold dose (0.003 GLE/FP) (Scheme 1).

The oviposition stimulant(s) in the aqueous layer was examined in combination with AA-I. The water layer was chromatographed on a reverse-phase column into four fractions, eluted with mixtures of acetic acid, methanol, and

SCHEME 1. Oviposition stimulant components for *Atrophaneura alcinous*.TABLE 1. OVIPOSITION RESPONSES OF *Atrophaneura alcinous* FEMALES TO *Aristolochia debilis* FRACTIONS ($N = 40$)

Sample	% Response (dose/filter paper)	
	0.03 gle	0.01 gle
Methanolic extract	100	100
Ether layer	8	0
Ethyl acetate layer	0	0
Butanol layer	8	0
Water layer (W)	75	23
W + Ether layer	—	93 ^a
W + Ethyl acetate layer	—	23
W + Butanol layer	—	33
W + Acidic fraction	—	90 ^a
W + Basic fraction	—	18
W + Neutral fraction	—	25

^aResponse significantly greater than that of the water layer alone (0.01 GLE) according to chi-square 2×2 test of independence with Yates' correction ($P < 0.01$).

water (Figure 2). Fraction W-1 exhibited weak but significant activity by itself at a 0.03 GLE dose, and its activity was markedly enhanced by adding AA-I (3 $\mu\text{g}/\text{FP}$) (Table 2). The active substance in W-1 was efficiently separated by a partition mode HPLC, as shown in Figure 3. Among the 11 fractions, only fraction 5 exhibited distinct activity in the presence of AA-I. The activity of fraction 6 appeared to be due to a tailing effect of the active peak in fraction 5. The pure active substance in the HPLC fraction was obtained in larger quantities by a combination of ion-exchange and charcoal columns, followed by recrystallization (mp 239–240°C). The content of the active compound was approximately 800 $\mu\text{g}/\text{GLE}$. The molecular formula of the compound was determined to be $\text{C}_7\text{H}_{14}\text{O}_6$ by an elemental analysis. It gave a pentaacetyl deriv-

ative (mp 197–198°C, m/z 404, in-beam MS). The [^1H]NMR [^{13}C]NMR spectra of both original and pentaacetyl compounds indicated the presence of an equatorial methoxy group attached to a cyclitol ring. The coupling constants in the [^1H]NMR spectra clearly provided the stereochemical relationship between adjacent methines. Thus the active compound was unequivocally assigned as sequoyitol (5-*O*-methyl-myoinositol) (Ohmoto et al., 1979; Binder and Had-don, 1984).

Figure 4 shows the dose responses of AA-I, sequoyitol, and a mixture of both in a leaf equivalent base, where the actual contents of AA-I and sequoyitol in the *A. debilis* extract were tentatively estimated to be 100 μg and 800 μg per gram of leaf, respectively. In contrast to AA-I alone, sequoyitol showed some stimulatory activity by itself at relatively high doses. When both were mixed together, it gave distinct activity and exhibited a typical dose–response curve. A 100% positive oviposition response was obtained at a dose of 0.1 GLE/FP or higher. However, the dose–response of the mixture still appeared to be lower than that of the original methanolic extract of *A. debilis*. The difference between the mixture (AA-I + sequoyitol) and the methanolic extract is considered to be

TABLE 2. OVIPOSITION RESPONSES OF *Atrophaneura alcinous* FEMALES TO COMBINATIONS OF FRACTIONS AND PURE COMPOUNDS

Sample (dose/filter paper)	% Response	(N)
Aristolochic acid (AA) I (10 μg)	6	(47)
AA B(10 μg)	6	(36)
Water layer (W) (0.003 gle)	19	(83)
AA I (10 μg) + W (0.003 gle)	84 ^a	(47)
AA B (10 μg) + W (0.003 gle)	64 ^a	(36)
AA I (3 μg)	8	(89)
W-1 (0.03 gle)	19	(21)
AA I (3 μg) + W-1 (0.03 gle)	91 ^b	(32)
AA I (3 μg) + W-2 (0.03 gle)	23	(32)
AA I (3 μg) + W-3 (0.03 gle)	13	(32)
AA I (3 μg) + W-4 (0.03 gle)	13	(32)
Sequoyitol (8 μg)	9	(32)
AA I (3 μg) + Sequoyitol (8 μg)	94 ^c	(32)
AA II (3 μg) + Sequoyitol (8 μg)	88 ^c	(32)
AA B (3 μg) + Sequoyitol (8 μg)	91 ^c	(32)
AA C (3 μg) + Sequoyitol (8 μg)	78 ^c	(32)
AA I (3 μg) + <i>myo</i> -Inositol (24 μg)	8	(32)

^aResponse significantly greater than that of the water layer alone.

^bResponse significantly greater than that of W-1 alone.

^cResponse significantly different both from aristolochic acid I (3 μg) alone and sequoyitol alone (chi-square 2 \times 2 test of independence with Yates' correction, $P < 0.01$).

partly due to the absence of other AA analogs besides AA-I in this bioassay series, but there are also some other additional factors present in the aqueous fraction (Nishida and Fukami, unpublished). The activity of AA-I, II, B, and C in combination with sequoyitol are summarized in Table 2. All of the AAs were shown to act significantly as synergists. Myoinositol did not induce the oviposition response in combination with AA-I, although it is one of the cyclitol constituents of *A. debilis* (approximately 800 $\mu\text{g}/\text{GLE}$).

DISCUSSION

Four analogs of aristolochic acids (AA-I, II, B, and C) and sequoyitol have been characterized here as oviposition stimulants for *A. alcinous*. Each individual component was inactive or only weakly active alone, but distinct oviposition activity was generated when either one of the AA analogs was mixed with sequoyitol. AAs are secondary metabolites contained characteristically in the genus *Aristolochia* (Mix et al., 1983). Considering the rare distribution of AAs in the plant kingdom, it would be an efficient chemical cue for the butterflies to recognize the host plant. In contrast to AAs, sequoyitol seems to be more commonly distributed in the plant kingdom, although it has been reported mostly from the Gymnospermae (Sato et al., 1966; Anderson et al., 1968, Cranswick and Zabkiwicz, 1979; Ohmoto et al., 1979; Binder and Haddon, 1984; Murakami et al., 1985). Sequoyitol is an *O*-methyl derivative of myoinositol, but myoinositol itself was found to be entirely inactive when admixed with AA-I, suggesting a fairly specific structural requirement. The simultaneous occurrence of AAs and sequoyitol in the plant seems to ensure an extremely high specificity for *A. alcinous* oviposition.

A. alcinous sequesters AAs in the body tissues or specialized organs throughout all stages from the egg to the adult (e.g., egg coating and larval osmeteria), and utilizes AAs as a defense against certain predators (Nishida and Fukami, 1989). AAs also synergize larval feeding activity (Nishida and Fukami, 1989). An assessment of the host-plant quality at both oviposition and larval feeding stages could guarantee consequent protection. Ecological adaptation of *A. alcinous* seems to be strongly associated with a sensory mechanism specifically developed for perception of AAs.

The chemical basis of oviposition behavior of papilionid butterflies has been extensively studied, particularly among the genus *Papilio*. A variety of flavonoid glycosides together with other polar components has been clarified as oviposition stimulant components of two Rutaceae-feeding swallowtail species, *Papilio xuthus* (Ohsugi et al., 1985; Nishida et al., 1987) and *P. protenor* (Honda, 1986). The importance of the multiple-component system has been emphasized in both cases. Feeny (1987) also reported a flavonoid as a key

substance of the oviposition stimulants for an Umbelliferae-feeding swallowtail, *P. polyxenes*. The contribution of the flavonoid components for *A. alcinous* oviposition, however, has not been clarified, although the butanol layer of *A. debilis* extracts contains a large quantity of rutin (600 $\mu\text{g}/\text{GLE}$), which is known as an oviposition stimulant for *P. xuthus* (Nishida et al., 1987).

More than 80 species in the family Papilionidae are associated with the family Aristolochiaceae. They are considered to represent remnants of the stock from which the rest of the Papilionidae evolved (Ehrlich and Raven, 1964). Seventy species of the tribe Troidini feed on Aristolochiaceae, but some (eight species) have been recorded from Rutaceae (Feeny et al., 1983). The oviposition stimulant, sequoyitol, is a cyclitol closely related to chiro-inositol that has been characterized as an oviposition stimulant component for *P. xuthus* (Ohsugi et al., unpublished). During the food-assessment phase of oviposition behavior, such underlying chemical similarity might have opened a new adaptive zone for the papilionid butterflies. A systematic survey of swallowtail oviposition stimulants may provide an evolutionary view of host specificity in the phylogenetic tree of the Papilionidae.

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(*S*)-2-PENTADECYL ACETATE AND 2-PENTADECANONE
Components of Aggregation Pheromone of
Drosophila busckii

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Abstract—(*S*)-2-Pentadecyl acetate and 2-pentadecanone were identified as the major aggregation pheromone components in *Drosophila busckii*. Both sexes of flies were attracted equally in a wind-tunnel olfactometer. The flies also responded to racemic 2-pentadecyl acetate but not to the pure *R* enantiomer. In bioassay, (*S*)-2-pentadecyl acetate and 2-pentadecanone were each active alone, and a mixture of both increased the number of flies responding ca. twofold. The aggregation pheromone components are found in the ejaculatory bulb of sexually mature males and are transferred primarily to the female cuticle during mating. One third of the pheromone transferred is released by the female to the surrounding environment in a few hours after mating. None of the aggregation pheromone components remained on the mated female's cuticle, leaving two thirds unaccounted for. The same results were obtained when racemic 2-pentadecyl acetate was topically applied to immature and mature virgin males and females. Both *D. mulleri* and *D. busckii* were attracted to (*S*)-2-acetates of 13, 14 and 15 carbons, but *D. mulleri* preferred (*S*)-2-tridecyl acetate and *D. busckii* preferred (*S*)-2-pentadecyl acetate.

Key Words—*Drosophila busckii*, *D. mulleri*, Diptera, Drosophilidae, aggregation, pheromone, (*S*)-2-pentadecyl acetate, 2-pentadecanone, chiral.

INTRODUCTION

Male-produced aggregation pheromones have been identified in a number of *Drosophila* species (Bartelt and Jackson, 1984; Bartelt et al., 1985a,b, 1986, 1988, 1989; Moats et al., 1987; Schaner et al., 1987, 1989a, b,c). Active compounds have included hydrocarbons, esters, and ketones. Mixtures of two or more compounds have often been required for optimum aggregation activity.

Drosophila busckii was chosen to continue *Drosophila* pheromone research since it is the only member of the subgenus *Dorsilopha* and we hoped to encounter new compounds that had not previously been identified as aggregation pheromones in other species studied. Here we report the identification, transfer, and bioassay characteristics of the aggregation pheromone of *D. busckii*.

METHODS AND MATERIALS

Flies. *D. busckii* (strain 13000-0081.0) was obtained from the National *Drosophila* Species Resource Center at Bowling Green, Ohio. They were originally collected at Heredia, Costa Rica. Flies were reared on Formula 4-24 Instant *Drosophila* Medium (Carolina Biological Supply House, Burlington, North Carolina) in 1-liter jars, 60-ml vials, or 10-ml test tubes under a 16 : 8 light-dark cycle and 23°–25°C.

Age of Maturity. Methods used to determine age of maturity of males and females have been described previously (Moats et al., 1987). Age of maturity of males is important because in other *Drosophila* species studied, pheromone production begins as the males approach sexual maturity. Male *D. busckii* become sexually mature at 2–3 days of age and females at 1–2 days of age. Therefore, 0 to 2-day-old flies were used for bioassays because the immature males would be less likely to produce pheromone that could compete with bioassay treatments. Six- to 7-day-old flies were used for extracts to ensure optimum levels of pheromone production.

Extracts and Chromatography. Flies were separated by sex when 0–16 hr old, maintained in 60-ml food vials to 6 to 7 days old, and extracted by soaking the flies in hexane at room temperature for 24 hr. The crude extracts were fractionated on open columns of silica gel (100–200 mesh; Bio-Rad) (Bartelt and Jackson, 1984) eluted with: hexane; 10% ether in hexane; 50% ether in hexane; and 10% methanol in methylene chloride.

Gas chromatography (GC) was conducted on a Varian 3700. For preparative GC, a 1.3-m × 4-mm 5% SE-52 column was used, and the effluent monitored with a thermal conductivity detector. A 15-m × 0.53-mm-ID Megabore DB-1 (J & W Scientific), and a 50-m × 0.2-mm-ID HP-1 narrow-bore capillary column (Hewlett-Packard) were utilized for analytical GC.

Electron impact mass spectra were obtained on a VG-MM16 mass spectrometer using a 30-m \times 0.25-mm-ID DB-5 capillary GC column for introduction of the sample.

Bioassay. Bioassays were conducted in a wind-tunnel olfactometer (23°–25°C) containing ca. 1000 flies (0–2 days old) that had been without food or water for approximately 2 hr (Bartelt and Jackson, 1984). An extract, fraction of an extract, synthetic compound, or control solvent was applied to a filter paper strip inserted around the lip of a glass vial. Each bioassay test consisted of placing two differently treated vials, to be compared, into the upwind end of the olfactometer for 3 min. When an experiment included more than two treatments, they were tested in pairs, in all possible combinations (a balanced incomplete block design). After 3 min, the vials were capped, removed, and the captured flies were sexed and counted. Tests were separated by 7–10 min, and as many as 50 tests could be run in a day after stocking the olfactometer once.

The bioassay data were transformed to the log ($X + 1$) scale before analysis to stabilize variance; analysis was done by the method of Yates (1940).

Production, Transfer, and Dispersal of Pheromone. Methods for the analysis of production of pheromone have previously been described (Schaner et al., 1989a). Briefly, ejaculatory bulbs were removed from male flies at various ages, extracted with hexane and analyzed by GC relative to an internal standard.

The transfer of (*S*)-2-pentadecyl acetate and 2-pentadecanone from males to females during mating and the subsequent release of the pheromone components from the females were investigated. Flies were separated by sex at 0–6 hr of age. Virgin male flies were raised individually in 10-ml test tubes that contained food medium. Virgin females were raised ca. 50 per 60-ml vial that contained food medium. At 6–7 days of age, when pheromone production for the male is optimum, a virgin female was aspirated without anesthesia into the test tube occupied by the virgin male where she remained until mating took place. After completion of mating, the male and female were separated, and in sets of three flies, either extracted or placed into empty 8-ml glass vials fitted with wire mesh caps for 6 hr and then extracted. Extraction consisted of removing the female reproductive tract or the male ejaculatory bulb and ducts, followed by a 5-min cuticular soak of the fly in 30 μ l of hexane. The glass vials used to evaluate recovery of any pheromone that had been released were rinsed with 500 μ l of hexane. Comparable sets of extracts were obtained from males and females that had not been allowed to mate. Each extract was replicated three times. The amount of pheromone components was quantified by GC relative to an internal standard of nonadecane.

Application of Racemic 2-Pentadecyl Acetate to Immature and Mature Virgin Males and Females. Immature virgin flies (2–3 hr) or mature virgin flies (6–8 days) were refrigerated for ca. 10 min to facilitate handling and placed in a Petri dish that was in direct contact with crushed ice. Using a 1.0- μ l syringe,

0.2 μ l of acetone containing 750 ng racemic 2-pentadecyl acetate for the immature flies or 550 ng racemic 2-pentadecyl acetate for the mature flies was applied to the posterior end of the fly's abdomen. Treated flies were put into empty 8-ml glass vials fitted with wire mesh caps (three flies per vial). At specific time intervals over a 6-hr period, three sets of flies were extracted. Extraction consisted of soaking the flies in 30 μ l of hexane for 5 min and rinsing the vials with 500 μ l of hexane. Each was analyzed using GC relative to an internal standard of nonadecane. Mature males possess (*S*)-2-pentadecyl acetate, therefore as a control, comparable extracts of mature males without racemic 2-pentadecyl acetate topically applied were obtained. The amount of (*S*)-2-pentadecyl acetate in each control extract was subtracted from the amount of racemic 2-pentadecyl acetate in the corresponding extract of males that had racemic 2-pentadecyl acetate applied to their cuticle.

Synthetic Ketone and Esters. Synthesis of the methyl ketone (2-pentadecanone) and esters [racemic 2-pentadecyl acetate, (*S*)-2-pentadecyl acetate, (*R*)-2-pentadecyl acetate and (*S*)-2-tetradecyl acetate] have been described in detail previously (Moats et al., 1987; Bartelt et al., 1989, and references therein). Methods were the same except tetradecanal was substituted for dodecanal when synthesizing racemic 2-pentadecyl acetate and 1-bromododecane or 1-bromoundecane was substituted for 1-bromodecane when reacted with the appropriate stereoisomer of lactate to synthesize (*S*)- and (*R*)-2-pentadecyl acetate or (*S*)-2-tetradecyl acetate. (*S*)-2-Tetradecyl acetate and (*S*)-2-tridecyl acetate (on hand from previous work with *D. mulleri*) were used in bioassay to test the flies' response to esters of various chain lengths with asymmetric centers. After silicic acid chromatography and preparative GC, the methyl ketone and synthetic esters were >98% pure.

Absolute Configuration. Diastereomeric esters from synthetic racemic, (*S*)-, (*R*)-, and male-derived 2-pentadecyl acetate were prepared as described previously (Bartelt et al., 1989, and references therein). Briefly, the acetates were reduced with LiAlH_4 to form the alcohol and derivatized with (*S*)-(2-acetyloxy)propanoyl chloride (Slessor et al., 1985). GC analysis was on a 50-m \times 0.2-mm-ID HP-1 narrow-bore capillary column (Hewlett-Packard) programmed from 150 $^\circ$ to 280 $^\circ$ C at 2 $^\circ$ /min.

RESULTS

Bioassay Characteristics. When the flies were first introduced into the olfactometer, they formed tight clusters in the upper corners with little movement and only a few in flight. Within 2 hr, the flies dispersed on the sides and back screen of the olfactometer, with ca. 20–30 flies in flight at any given time. Response to an attractive treatment included an increase in the number of flies

in flight, an upwind zigzag flight to the vial, hovering within a few inches of the vial, alighting on or near the vial, and walking to the lip of the vial where the majority proceeded down the inside of the vial.

Identification of Pheromone Components. The crude extract of male *D. busckii* was clearly active in bioassay, while the crude extract of females was inactive (Table 1). Both sexes responded similarly to all preparations discussed in this report.

After chromatography of the crude extract of males on silicic acid, only the 10% ether-hexane fraction was substantially more active than controls (Table 1). The 10% ether-hexane fraction from males contained only two detectable compounds as analyzed by capillary GC. From retention times of aggregation pheromone components of *D. mulleri* and *D. hydei* (Bartelt et al., 1989; Moats et al., 1987), the first of the two compounds (287 ng/fly) was tentatively identified as 2-pentadecanone. Gas chromatography-mass spectra (GC-MS) confirmed this identification. Mass spectra of the second compound (1067 ng/fly) appeared similar to mass spectra of (*S*)-2-tridecyl acetate, an aggregation pheromone component of *D. mulleri* (Bartelt et al., 1989), except it was two carbons greater in chain length. Synthetic racemic 2-pentadecyl acetate had identical GC retention times (DB-1) and mass spectra. Racemic 2-pentadecyl acetate was active in bioassay (Table 2, A).

The fly-derived 2-pentadecyl acetate, with one asymmetric center, was analyzed for absolute configuration. The two diastereomers derived from racemic 2-pentadecanol were easily separated on the HP-1 narrow-bore capil-

TABLE 1. ACTIVITY OF SILICA GEL FRACTIONS AND EXTRACTS RELATIVE TO CRUDE EXTRACT OF MALE *D. busckii*

Extract or fraction ^a	Relative response (<i>N</i> ≥ 8) ^b
Crude extract of males	100*** ^c
Hexane fraction	5 (3.0, 4.0, 23.5) ^d
10% Ether-hexane fraction ^e	92***(0.9, 38.3, 41.5)
50% Ether-hexane fraction	2 (0.7, 1.1, 17.5)
10% Methanol-methylene chloride	19* (6.5, 16.0, 56.5)
Crude extract of females	2 (0.4, 0.7, 16.0)

^a All extracts and fractions were used at one fly equivalent per test.

^b Relative response = (fraction - control)/(crude extract of males - control) × 100.

^c 100 by definition. * and *** denote significance of *t* tests vs. controls at the 0.05 and 0.001 levels, respectively.

^d Mean bioassay catches for control, treatment, and crude extract of males, respectively, shown in parentheses.

^e Because 10% and 50% ether-hexane solvents were attractive, these fractions were taken to dryness under nitrogen and back up in hexane.

TABLE 2. BIOASSAY RESULTS LEADING TO IDENTIFICATION OF ACTIVE COMPOUNDS IN 10% ETHER-HEXANE FRACTION FROM MALE *D. busckii*

Treatment	Mean bioassay catch
A. Preliminary identification of ester component ($N = 12$)	
Control	4.5a ^a
Racemic 2-pentadecyl acetate (1000 ng)	34.9b
B. Determination of active enantiomer ($N = 12$).	
Control	0.7a
(<i>R</i>)-2-Pentadecyl acetate (1000 ng)	0.9a
(<i>S</i>)-2-Pentadecyl acetate (1000 ng)	11.1b
C. Synergism of synthetic ester and ketone ($N = 12$) (ketone and ester were used at 287 and 1067 ng, respectively, per test)	
Control	0.4a
2-Pentadecanone	2.6b
(<i>S</i>)-2-Pentadecyl acetate	9.5c
(<i>S</i>)-2-Pentadecyl acetate + 2-pentadecanone	24.1d
D. Comparison of the male 10% ether-hexane fraction and synthetic ester and ketone ($N = 12$) [both treatments had 1068 ng (<i>S</i>)-2-pentadecyl acetate and 287 ng 2-pentadecanone, ca. 1 fly eq.]	
Control	0.2a
(<i>S</i>)-2-Pentadecyl acetate + 2-pentadecanone	22.1b
Male 10% ether-hexane fraction	17.1b

^aWithin each subheading, means followed by the same letter were not significant at the 0.05 level (LSD). Comparison of means between subheadings are not meaningful because the experiments were done on different days, with different groups of flies.

lary column. Based on the retention times of synthetic standards, the first of these peaks corresponded to the *R* configuration while the second peak corresponded to the *S* configuration. The derivative prepared from the *D. busckii* pheromone component corresponded only to the *S* configuration. *D. busckii* responded in bioassay only to the (*S*)-2-pentadecyl acetate; whereas (*R*)-2-pentadecyl acetate was inactive (Table 2, B).

Dose Study of S-2-Pentadecyl Acetate. Doses of 100 and 1000 ng of (*S*)-2-pentadecyl, along with a control, were compared in bioassay. The mean catches for control, 100 ng (*S*)-2-pentadecyl acetate, and 1000 ng (*S*)-2-pentadecyl acetate were 1.8, 4.7, and 26.0, respectively ($N = 16$). The 100-ng dose of (*S*)-2-pentadecyl acetate was significantly more attractive to the flies than the control, but the response was subtle.

Synergistic Activity of S-2-Pentadecyl Acetate and 2-Pentadecanone. (*S*)-2-Pentadecyl acetate and 2-pentadecanone were active alone in bioassay; however, a two fold increase in the mean number of flies caught was observed when

the ester and ketone were combined (Table 2, C). The combination of (*S*)-2-pentadecyl acetate and 2-pentadecanone accounted for all the activity of the 10% ether-hexane fraction from males (Table 2, D).

Aggregation Activity Specificity to S-2-Acetates of 13, 14, and 15 Carbons. When synthetic (*S*)-2-tridecyl acetate, (*S*)-2-tetradecyl acetate, and (*S*)-2-pentadecyl acetate, along with a control, were compared in bioassay (Table 3), both *D. busckii* and *D. mulleri* responded best to the (*S*)-2-acetate ester they produce. Both species responded to all of the (*S*)-2-acetate esters significantly over controls. The attraction towards the (*S*)-2-acetate esters decreased as the number of carbons increased away from the conspecific acetate ester.

Location and Production of 2-Pentadecanone and S-2-Pentadecyl Acetate. As observed in other *Drosophila* species (Brieger and Butterworth, 1970; Schaner et al., 1987, 1989a, b, c), the ejaculatory bulb of *D. busckii* mature males was the site of storage of the aggregation pheromone components. (*S*)-2-Pentadecyl acetate and 2-pentadecanone increased in concentration from 0 ng/fly at 0-4 hr to a plateau of 1100 and 250 ng/fly, respectively, at day 4 (Figure 1).

Transfer and Dispersal of Aggregation Pheromone Components. Virgin females of any age did not possess (*S*)-2-pentadecyl acetate or 2-pentadecanone (Table 4, A). In mature mated or virgin males, 90% of the aggregation pheromone components are found in the ejaculatory bulb, while 10% are found on the surface of the fly. During mating, males transferred approximately one third of their quantity of aggregation pheromone components to the female. Ninety percent of the (*S*)-2-pentadecyl acetate and 2-pentadecanone was transferred to the female cuticle and only 10% was transferred to the reproductive tract.

TABLE 3. SPECIFICITY FOR ACETATE ESTER STRUCTURE IN AGGREGATION BIOASSAY FOR *D. busckii* AND *D. mulleri*.

Treatment ^a	Mean bioassay catch (N = 12)	
	<i>D. busckii</i>	<i>D. mulleri</i>
Control	0.2a ^b	3.8a
(<i>S</i>)-2-Tridecyl acetate	2.3b	27.3c ^c
(<i>S</i>)-2-Tetradecyl acetate	6.2c	20.4b
(<i>S</i>)-2-Pentadecyl acetate	15.4d ^c	15.4b

^aThe acetate ester treatments were 1000 ng for *D. busckii* and 300 ng for *D. mulleri*.

^bMeans followed by the same letter were not significantly different at the 0.05 level (LSD). Comparisons of means between columns are not meaningful because the experiments were done on different days, with different species of flies.

^cDenotes male-specific acetate ester.

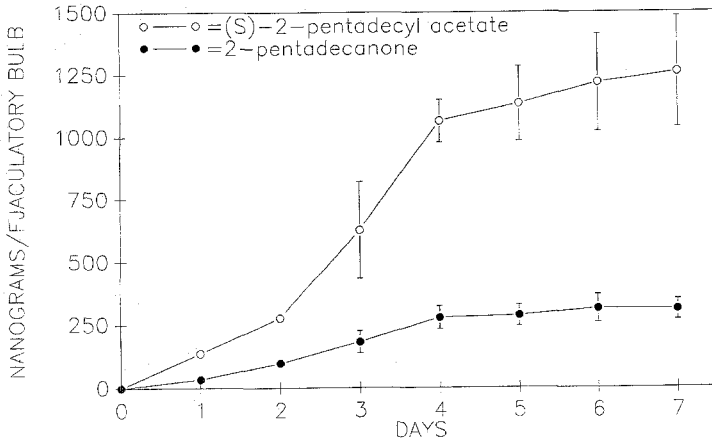


FIG. 1. Pheromone content in the ejaculatory bulb of male flies as they age.

Within 6 hr after completion of mating, approximately one third of the quantity of aggregation pheromone components transferred to the female could be found in the holding vial with none remaining on the female, leaving two thirds unaccounted for (Table 4, B). When a known amount of racemic 2-pentadecyl acetate was deposited in a vial fitted with a wire mesh cap for 6 hr, 91% was recovered. The amount of (*S*)-2-pentadecyl acetate and 2-pentadecanone the female emitted to the holding vial, however, was three times more than a mated or virgin male emitted to the holding vial in a 6-hr period. Six hours after mating, males still had the same quantity of (*S*)-2-pentadecyl acetate (ca. 100 ng/fly) and 2-pentadecanone (ca. 25 ng/fly) on their body surface as virgin males or males that had just finished mating. Also, males that had mated recently or 6 hr previously had the same quantity of the aggregation pheromone components in their ejaculatory bulbs.

Application of Synthetic Racemic 2-Pentadecyl Acetate to Immature and Mature Virgin Males and Females. To acquire some more information on the disappearance of (*S*)-2-pentadecyl acetate, synthetic racemic 2-pentadecyl acetate in acetone was applied to the cuticle of immature and mature virgin males and females. Surface extracts of the flies and a rinse of their holding vials were obtained and analyzed. Racemic 2-pentadecyl acetate rapidly disappeared from the surface of all the flies at virtually the same rate. Within the first hour, racemic 2-pentadecyl acetate rapidly disappeared and the decrease was more gradual for the next 3 hr (Figure 2). After 4 hr, the applied racemic 2-pentadecyl acetate was nearly gone from the cuticle of all the flies. The holding vials contained 20–30% of the applied racemic 2-pentadecyl acetate, and the amount deposited was independent of time.

TABLE 4. TRANSFER OF AGGREGATION PHEROMONE COMPONENTS FROM MALES TO FEMALES DURING MATING AND RELEASE OF PHEROMONE BY BOTH *D. busckii* SEXES

Source	Pheromone (ng/fly, mean \pm SD)	
	2-Pentadecanone	(<i>S</i>)-2-Pentadecyl acetate
A. Before or just after mating		
Virgin male		
Ejaculatory bulb	351 \pm 63	1056 \pm 187
Remainder of fly ^a	27 \pm 3	118 \pm 31
Mated male		
Ejaculatory bulb	211 \pm 12	647 \pm 57
Remainder of fly	31 \pm 7	103 \pm 22
Virgin female		
Reproductive tract	0	0
Remainder of fly	0	0
Mated female		
Reproductive tract	1 \pm 0	40 \pm 4
Remainder of fly	116 \pm 28	403 \pm 59
B. Six hours after mating		
Virgin male		
Ejaculatory bulb	319 \pm 90	1097 \pm 140
Remainder of fly	24 \pm 5	111 \pm 34
Vial ^b	trace	21 \pm 15
Mated male		
Ejaculatory bulb	184 \pm 11	602 \pm 47
Remainder of fly	18 \pm 4	92 \pm 18
Vial	16 \pm 3	37 \pm 1
Mated female		
Reproductive tract	3 \pm 4	23 \pm 7
Remainder of fly	2 \pm 3	4 \pm 4
Vial	50 \pm 8	120 \pm 32

^aFive-minute hexane extract of remainder of fly with reproductive tract or ejaculatory bulb removed.

^bExtract of glass vial that flies spent 6 hr.

DISCUSSION

Drosophila busckii have a male-produced aggregation pheromone comprised of two major components identified as (*S*)-2-pentadecyl acetate and 2-pentadecanone. (*S*)-2-Pentadecyl acetate was the most abundant component and the most active in bioassay. (*S*)-2-Pentadecyl acetate is homologous to (*S*)-2-tridecyl acetate, an aggregation pheromone component in *D. mulleri* (Bartelt et al., 1989). This is not the first observation of compounds of a homologous

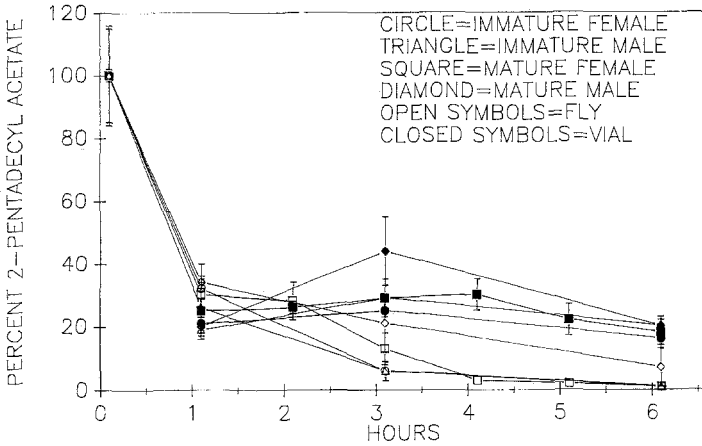


FIG. 2. Application of synthetic racemic 2-pentadecyl acetate to immature and mature virgin males and females.

series functioning as aggregation pheromone components in different species of *Drosophila*. In the *melanogaster* group, members of the *melanogaster* subgroup use (*Z*)-11-octadecenyl acetate as their aggregation pheromone (Bartelt et al., 1986; Schaner et al., 1987; Schaner et al., 1989c) and members of the *bipunctinata* complex of the *ananassae* subgroup use (*Z*)-11-eicosenyl acetate, which differs in length by two carbons, as their aggregation pheromone (Schaner et al., 1989a, b). 2-Pentadecanone had previously been identified as a minor component of the aggregation pheromone in *D. hydei* (Moats et al., 1987). This is, however, the first observation of (*S*)-2-pentadecyl acetate and 2-pentadecanone produced by a single species and acting as coattractants.

In most of the *Drosophila* species studied to date, the aggregation pheromone components were transferred predominantly to the reproductive tract of the female. In *D. busckii*, however, a major portion of the (*S*)-2-pentadecyl acetate and 2-pentadecanone was transferred onto the surface of the female while only a small portion was found in the reproductive tract. The timing and mechanism of transfer of the aggregation pheromone components from male to female is unknown in *D. busckii*. It does not appear that the transfer is from cuticle to cuticle since the content on the cuticle of males remained nearly constant while there was a dramatic increase on the cuticle of the female.

In just a few hours after copulation, the mated female *D. busckii* no longer has pheromone components on her cuticle. The mated female appears to have transferred a small portion of pheromone components from her cuticle to her surroundings. This is most likely a major process in the release of the pheromone to cause aggregation at or near sites where mating has taken place (Bartelt et al., 1984). The majority of the transferred (*S*)-2-pentadecyl acetate and 2-

pentadecanone disappeared and was not available to stimulate aggregation. Synthetic racemic 2-pentadecyl acetate disappears at the same rate when topically applied to immature and mature virgin males and females. The vapor pressure for the pheromone compounds is too low to account for the loss due to evaporation. It would appear that the cuticle of both female and male *D. busckii* have enzymes that catabolize the pheromone components so that only recently mated females are appreciable emitters of the pheromone.

Catabolism of acetate esters by enzyme(s) on the cuticle has been observed in other species of *Drosophila*. In *D. funebris*, (*Z*)-11-octadecenyl acetate was transferred from the ejaculatory bulb of the male primarily to the female cuticle during mating, where it was rapidly catabolized by the cuticle of the female (Skiba et al., submitted). *D. melanogaster*, *D. virilis*, *D. americana*, *D. buzzatii*, *D. limpiensis*, and *D. macrospina* (Skiba, et al., submitted) virgin females all catabolize (*Z*)-11-octadecenyl acetate (1000 ng in acetone) applied to the posterior end of the abdomen with at least 90% catabolism in 8 hr. This would suggest that mated females only emit the pheromone to the surroundings for a few hours after mating and acetate ester components of the pheromone that are on the cuticle of the abdomen are catabolized to limit the time that a female can emit the pheromone.

The types of compounds (i.e., ketone and acetate ester of a secondary alcohol) that comprise the pheromone of *D. busckii* are like the compounds previously identified as aggregation pheromones in species of the *repleta* group. *D. busckii* employs a two-component blend more like the aggregation pheromone from *D. mulleri*, which employs (*Z*)-10-heptadecen-2-one and (*S*)-2-tridecyl acetate (Bartelt et al., 1989), than *D. hydei*, which employs 2-tridecanone, 2-pentadecanone, and tiglate esters (Moats et al., 1987). The transfer of aggregation pheromone components from male to female *D. busckii* is more like the transfer in *D. funebris* where the pheromone components appear on the cuticle of the female. *D. busckii*, while phylogenetically separated in the *Dorsilopha* subgenus, utilizes biochemical and biological strategies to promote aggregation that are similar to species in the *Drosophila* subgenus, utilizes biochemical and biological strategies to promote aggregation that are similar to species in the *Drosophila* subgenus.

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CHEMISTRY OF MANDIBULAR AND DUFOUR'S
GLAND SECRETIONS OF ANTS IN GENUS
Myrmecocystus

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Abstract—Males of several species of *Myrmecocystus* produce mandibular gland secretions that contain 2,4-dimethyl-2-hexenoic acid and a variety of monoterpenes that include neral, geranial, citronellol, limonene, and 2,6-dimethyl-5-hepten-1-ol. Other components identified include methyl anthranilate, octanal, octanol, octyl octanoate, and 2-hexyl-2-decenal. Methyl salicylate has been identified as a mandibular gland constituent of workers of several species in addition to mellein and monoterpenes such as cymene, limonene, and the isomers of citral. The Dufour's gland secretions of workers and females of 14 species contain typical formicine alkanes (e.g., undecane), 2-alkanols (e.g., 2-tridecanol), and 2-alkanones (e.g., 2-tridecanone). Two species in the subgenus *Eremnocystus* produce secretions that are distinguished by the presence of significant quantities of tridecyl esters. The functions of these compounds as well as their possible chemosystematic significance in the genus *Myrmecocystus* are discussed.

Key Words—*Myrmecocystus* species, Hymenoptera, Formicidae, mandibular glands, Dufour's gland, chemosystematics, sex pheromones, defensive allomones, methyl anthranilate, citral, methyl salicylate, tridecyl esters.

INTRODUCTION

Honey ants in the small genus *Myrmecocystus* are characteristic of the arid and semiarid habitats of western North America. In terms of number of species, this is the only successful formicine genus in the North American deserts, 28 species having been described from these xeric communities (Snelling, 1976, 1982). *Myrmecocystus* species are generally omnivorous, exploiting carbohydrate-rich sources from plants and insects as food, in addition to being predators and scavengers. Workers of many species function as a honeypot caste, storing large amounts of concentrated carbohydrate solutions in their filled crops that are accommodated by their greatly expanded gasters. In addition to this distinctive behavior, some species maintain territories by engaging in elaborate display tournaments with nearby conspecific colonies that often result in nest raiding (Hölldobler, 1976).

In view of the recent revision of the genus *Myrmecocystus* (Snelling, 1976, 1982), it is an especially appropriate time to undertake a comparative investigation of the exocrine chemistry of the species in this taxon. In the present report we describe the mandibular gland chemistry of members of different castes of several species of *Myrmecocystus*. In addition, the Dufour's gland chemistry of females and/or workers are described for 14 species in this genus.

METHODS AND MATERIALS

Ants

Collections were made at the sites listed below.

Subgenus Myrmecocystus. *M. christinae*: 9.5 mi NNE Cima, San Bernardino County, California; *M. mexicanus*: 9.5 mi NNE Cima, San Bernardino County, California and 2 mi S Pearblossom, Los Angeles County, California; *M. navajo*: 15 mi W Albuquerque, Bernalillo County, New Mexico and 23 mi SW Silver City, Grant County, New Mexico; *M. testaceus*: Jackass Springs, Inyo City, California.

Subgenus Endiodioctes. *M. mendax*: 9.5 mi NNE Cima, San Bernardino County, California, 8.1 mi SE Sunnyside, Cochise County, Arizona, and 6 mi W Adrian, Oldham County, Texas; *M. depilis*: 11.4 mi W Las Cruces, Dona Ana Co., New Mexico, and Jornada Experimental Range, Dona Ana County, New Mexico; *M. mimicus*: 2 mi S Pearblossom, Los Angeles County, California; *M. romainei*: 9.5 mi NNE Cima, San Bernardino County, California, Jackass Springs, Inyo City, California, Jornada Experimental Range, Dona Ana County, NM, 15 mi W Albuquerque, Bernalillo County, New Mexico, and 10 mi SE El Paso County, Texas; *M. semirufus*: 2 mi S Pearblossom, Los Angeles

County, California; *M. wheeleri*: 6 mi SE Pearblossom, Los Angeles County, California; *M. kennedyi*: 5 mi N Wadsworth, Washoe County, Nevada; *M. kathjuli*: 2 mi S Pearblossom, Los Angeles County, California; *M. intonsus*: 17 mi E Todos Santos, Baja California Sur, Mexico; *M. flaviceps*: 3 mi E Thousand Palms, Riverside County, California.

Subgenus Eremnocystus. *M. colei*: 7 mi NW Cajon, San Bernardino County, CA; *M. creightoni*: 2 mi S Pearblossom, Los Angeles, County, California.

Specimens of all species have been deposited in the Natural History Museum of Los Angeles County.

Fragments of colonies of *M. mexicanus* were maintained in laboratory nests for testing of glandular secretions or identified compounds. Nests contained 200–300 workers and were fed honey water and insects. Mandibular and Dufour's glands (plus reservoirs) were dissected from frozen workers and crushed on sharpened match sticks, which were presented within 2 cm of the nest entrance. The reactions of workers were observed for 60 sec. In addition, the effects of synthetic compounds (2 μg) were evaluated after applications to 1-cm filter paper disks as 1- μl pentane solutions. Controls consisted of pentane applied to disks.

Chemistry

Extracts of Dufour's and mandibular glands were prepared by dissecting these organs from frozen workers under water and crushing the glands in a small volume of pentane. In addition, concentrated extracts of these glands were prepared from extracts of heads and gasters in pentane.

The extracts were concentrated and examined by GC-MS on an LKB-9000 or an LKB-2091 instrument utilizing the following stationary phases: 1.86 m 1% OV-17 programmed from 60° to 300°C at 10°/min; 3.7 m 10% SP-1000 programmed from 50° to 220°C at 10°/min; 25 m SE-30 capillary column programmed from 50° to 320°C at 10°/min.

Structural assignments were based on comparisons of mass spectra and GC retention times of authentic standards with glandular constituents. Standards were obtained from commercial sources or were synthesized. Octyl octanoate was synthesized from octyl alcohol and octanoyl chloride. The tridecyl esters were prepared from tridecanol and the appropriate acid chlorides.

RESULTS

Chemistry of Mandibular Gland Secretions of Males. The volatile constituents in the mandibular glands of males of six species of *Myrmecocystus* were analyzed (Table 1). Four of these species, *M. depilis*, *M. romainei*, *M. semi-*

TABLE 1. MANDIBULAR GLAND CONSTITUENTS OF MALES OF *Myrmecocystus* SPECIES

Compound ^a	Subgenus and species					
	(<i>Myrmecocystus christinae</i>)	(<i>Eremmocystus cole^b</i>)	<i>depilis^c</i>	<i>romainei^d</i>	<i>semirufus</i>	<i>wheeleri^b</i>
Citronellol	+					
2,6-Dimethyl-5-hepten-1-ol		++		++		+
2,4-Dimethyl-2-hexenoic acid	++	++	++	++	++	++
Geraniol						
2-Hexyl-2-decenal			+	+		
Limonene		+				
Methyl anthranilate				+	+	
Neral					++	
Octanal			++	++		
Octanol		++	++	+		
Octyl octanoate		++	++	++		

^a Minor and major compounds indicated by one and two pluses, respectively.

^b Produces an unidentified monoterpene alcohol as a major constituent.

^c One population lacks methyl anthranilate but produces 2-hexyl-2-decenal.

^d Only octanol and 2,4-dimethyl-2-hexenoic acid detected in one population; 2,4-dimethyl-2-hexenoic acid not detected in two populations.

rufus, and *M. wheeleri* are members of the subgenus *Endiodioctes*, the largest and most widely distributed of the three subgenera of *Myrmecocystus* (Snelling, 1976). *M. christinae*, on the other hand, is a member of the subgenus *Myrmecocystus*, whereas *M. colei* is a taxon in the subgenus *Eremnocystus*.

The only compound that is common to the secretions of all six species is 2,4-dimethyl-2-hexenoic acid (Table 1). Several monoterpenoids are concomitants of this acid, and in the cases of *M. christinae* and *M. wheeleri* they constitute the only other volatile compounds detected. The secretion of *M. christinae* is distinguished by the presence of citronellol, whereas that of *M. wheeleri* contains 2,6-dimethyl-5-hepten-1-ol and an unidentified monoterpene alcohol that possesses a mass spectrum similar to that of citronellol. *M. colei* also produces this monoterpene alcohol as well as limonene and the dimethylheptenol. The exudates of *M. semirufus* are distinctive in containing two other monoterpenes, the geometric isomers of citral: neral and geranial.

Males of *M. depilis* and *M. romainei* produce secretions that are dominated by octanol, octanal, and octyl octanoate; 2-hexyl-2-decenal is a minor component in the secretions of both species. Males of one population of *M. romainei* are also distinctive in synthesizing methyl anthranilate, the only aromatic constituent detected in these male exudates. This ester is also produced by males of *M. semirufus* (Table 1).

Chemistry of Mandibular Gland Secretions of Workers and Females. Volatile compounds were identified in the mandibular gland secretions of females of one species and workers of five other species of *Myrmecocystus*. As was the case with the male secretions, monoterpenes were characteristic components in the secretions of most of the species.

Females of *M. creightoni* produced a distinctive secretion that contains two monoterpene hydrocarbons, cymene and limonene (Table 2). On the other hand, the glandular exudate of workers of *M. mendax* contains neral and geranial, as does the secretion of males of this species (Table 1).

By contrast, the secretions of workers of *M. kathjuli* and *M. mexicanus* contain a single detectable volatile, the aromatic ester methyl salicylate. This compound also dominates the citral-rich secretions of workers of *M. semirufus* and *M. testaceus* (Table 2). One population of *M. testaceus* was particularly distinctive in producing the lactone mellein. Volatiles could not be detected as mandibular gland products of workers of eight other species of *Myrmecocystus*. Only structural lipids were evident in analyses of extracts of either mandibular glands or heads of *M. christinae*, *M. depilis*, *M. flaviceps*, *M. intonsus*, *M. kennedyi*, *M. mimicus*, *M. navajo*, and *M. wheeleri*.

Chemistry of the Dufour's Gland Secretions of Workers and Females. Dufour's gland constituents were identified from workers of 14 species of *Myrmecocystus* as well as females of three of these species (Table 3). Dufour's

TABLE 2. VOLATILE COMPOUNDS OF MANDIBULAR GLANDS OF FEMALES AND WORKERS OF *Myrmecocystus* SPECIES

Compound	Subgenus and species					
	<i>(myrmecocystus)</i> <i>mexicanus</i> ^a	<i>(Eremnocystus)</i>		<i>(Endiodioctes)</i>		
		<i>testaceus</i> ^a	<i>creightoni</i> ^b	<i>kathjuli</i> ^b	<i>mendax</i> ^b	<i>semirufus</i> ^b
Cymene			+			
Geranial		+			+	+
Limonene			+			
Mellein		+				
Methylsalicylate	+			+		+
Neral		+			+	+

^a Only workers were analyzed.

^b Only detected in females.

^c Only detected in one population of workers.

gland chemistry of females was comparable to that of their corresponding workers.

The alkanes undecane and tridecane are characteristic of the secretions of all species, the former compound invariably being a major constituent. Although C₁₀, C₁₂, and C₁₅ alkanes are produced by some species (Table 3), these compounds constitute minor glandular products at best.

2-Tridecanol is a glandular product detectable in only four species; higher 2-alkanols have only been identified in the secretion of *M. mexicanus*. On the other hand, the methyl ketones 2-tridecanone and 2-pentadecanone are synthesized by most of the species; 2-heptadecanone and 2-nonadecanone are present in the exudates of only a few species (Table 3).

Tridecyl esters distinguish the Dufour's gland secretions of *M. colei* and *M. creightoni* from those of the other *Myrmecocystus* species. A single ester, tridecyl decanoate dominates the exudate of *M. colei*, and this compound is also the major volatile compound in the secretion of *M. creightoni*. However, in addition, the latter species produces tridecyl octanoate, nonanoate, undecanoate, and dodecanoate.

Phenol, a minor constituent, in the secretions of *M. mendax*, *M. navajo*, *M. romainei*, and *M. semirufus*, (Table 3), is the only aromatic compound identified as a Dufour's gland product of *Myrmecocystus* species.

Behavioral Effects of Exocrine Compounds. Crushed mandibular glands of *M. mexicanus* (10 tests) cause excited movements in the nearby ants, which frequently are attracted to the pheromonal source. Workers exhibit jerky movements and sometimes elevate their legs. Additional alerted workers from the

TABLE 3. DUFOUR'S GLAND CONSTITUENTS OF *Myrmecocystus* WORKERS AND FEMALES

Subgenus and species	Compound													
	Alkanes			2-Alkanols				2-Alkanones				Tridecyl esters	Phenol	
	C ₁₁	C ₁₃	C ₁₅	C ₁₃	C ₁₅	C ₁₇	C ₁₃	C ₁₅	C ₁₇	C ₁₉				
<i>(Myrmecocystus)</i>														
<i>christinae</i> ^a	+	+					+							
<i>mexicanus</i> ^{b,c}	+	+	+		+		+							
<i>navajo</i> ^{b,d}	+	+					+							
<i>testaceus</i> ^a	+	+					+							+
<i>(Eremmocystus)</i>														
<i>colei</i> ^b	+	+												
<i>creightoni</i> ^{b,d}	+	+												
<i>(Endiodioctes)</i>														
<i>flaviceps</i> ^a	+	+					+							
<i>intonsus</i> ^b	+	+					+							
<i>kathijuli</i> ^b	+	+					+							
<i>kennedyi</i> ^b	+	+					+							
<i>mendax</i> ^a	+	+					+							
<i>mimicus</i> ^b	+	+					+							+
<i>romainei</i> ^{a,e}	+	+					+							+
<i>semirufus</i> ^{b,e}	+	+					+							+

^aWorkers and females.^bOnly workers analyzed.^cDecane and pentadecane also present.^dDecane and undecene also present.^eDodecane also present.

nest are attracted to the emission source, and they frequently challenge nest-mates momentarily. Methyl salicylate produces very similar reactions in the workers.

Crushed Dufour's glands generally produce repellency ($N = 10$), the workers moving frenetically from the glandular source, often into the nest. Excited workers sometimes bolt from the nest. Mixtures of 2-tridecanol and 2-tridecanone ($N = 10$) produce reactions similar to those obtained with crushed Dufour's glands, but the addition of the other 2-alkanols and 2-alkanones in the secretion (Table 3) does not appear to appreciably augment the activity of the binary mixture.

DISCUSSION

The exocrine chemistry of species in the genus *Myrmecocystus* is clearly consistent with that of species in several taxa in the subfamily Formicinae. Analyses of the formicine genera *Lasius* (Bergström and Löfquist, 1970), *Acanthomyops* (Chadha et al., 1962; Regnier and Wilson, 1968), *Formica* (Bergström and Löfquist, 1973), and *Camponotus* (Brand et al., 1973a,b; Duffield, 1976) have demonstrated that characteristic classes of compounds are biosynthesized in common in their mandibular and Dufour's glands. In particular, the suggestion that *Myrmecocystus* is allied with the genera in tribe Lasiini (Snelling, 1976) is consistent with the natural product emphases observed for these taxa.

For example, males in the lasiine genera *Acanthomyops* and *Lasius* synthesize 2,6-dimethyl-5-hepten-1-ol in their mandibular glands (Law et al., 1965), as do *Myrmecocystus colei* and *M. wheeleri*. The identification of seven terpenes as mandibular products of *Myrmecocystus* species (Tables 1 and 2) is paralleled by the demonstration that *Lasius* species produce a variety of terpenes in these organs (Bergström and Löfquist, 1970; Blum et al., 1968). Monoterpenes such as neral, geranial, and citronellol are commonly produced by *Lasius* species (Bernardi et al., 1967; Law et al., 1965) as they are by *Myrmecocystus* species. Even the nonterpenoid octanol, a product of males of *M. depilis* and *M. romainei*, has been identified as a mandibular gland constituent of *L. niger* (Bergström and Löfquist, 1970).

On the other hand, the monoterpene hydrocarbons cymene, a product of females of *M. creightoni*, and limonene, a glandular constituent of females of *M. creightoni* and males of *M. colei*, have not been previously identified as a formicid mandibular gland products. It may be significant that these terpenoids have only been identified as exocrine products of two species in the subgenus *Eremnocystus*.

While terpenes appear to be widely distributed in the mandibular gland

secretions of males of *Myrmecocystus* species, a nonterpenoid, 2,4-dimethyl-2-hexenoic acid, is the most widespread compound identified in these male exudates. This acid has been previously identified as a mandibular gland product of males of several *Camponotus* species, particularly those in the subgenus *Myrmentoma* (Brand et al., 1973a; Duffield, 1976). Indeed, *Camponotus* males in this subgenus also produce methyl anthranilate (Brand et al., 1973a; Duffield, 1976), a glandular constituent synthesized by males of both *M. romainei* and *M. semirufus*, two species in the subgenus *Endiodioctes*. The blend of compounds synthesized by males of some populations of *M. romainei* is further distinguished by the presence of octyl octanoate, octanal, and octanol, an aliphatic trio which they share with males of *M. depilis*.

Methyl anthranilate may be chemotaxonomically significant for *M. romainei*. This compound is a glandular constituent of males of one California population of this species. On the other hand, the secretions of males from west Texas populations do not contain detectable amounts of this ester but rather, in its place, 2-hexyl-2-decenal, which represents the aldol condensation-dehydration product of octanal. This difference in chemical characters parallels morphological differences between major workers in the two populations, and one of us (R.R.S.) is analyzing the possible significance of these incongruities.

Although *Camponotus* males are not distinguished as producers of terpenes, one species, *C. clarithorax*, excels in the biosynthesis of these compounds. One of the major terpenes produced by this species is 2,6-dimethyl-5-hepten-1-ol, which is accompanied by several other monoterpenes (Lloyd et al., 1975). This compound is also a major product of males of two *Colobopsis* species, which produce other monoterpenes (e.g., citronellol) as well (Duffield, 1976). If males of *Colobopsis* species generally synthesize terpenes, they will join *Myrmecocystus* and lasiine species as characteristic producers of terpenoid pheromones.

Hölldobler and Maschwitz (1985) demonstrated that the sex-specific secretions of *Camponotus* males function as sex pheromones. Suffice it to say that the compounds produced by *Myrmecocystus* males, several of which are shared with *Camponotus* males, seem eminently suited to be utilized as sex pheromones for honey ant species.

Methyl salicylate, a mandibular gland product of workers of *M. kathjuli*, *M. mexicanus*, and *M. semirufus*, has not previously been identified as a natural product of ants. Indeed, this ester is only known as an exocrine product of one other arthropod, the carabid beetle *Idiochroma dorsalis* (Schildknecht et al., 1968). Our laboratory investigations indicate that this compound functions as an alarm pheromone for workers of *M. mexicanus*, the odor of this ester being readily evident when the ants are disturbed.

Although it is not unlikely that sex-specific mandibular gland products (=sex pheromones) are characteristic of males of *Myrmecocystus* species, the

same cannot be said for the workers or females. Notwithstanding the fact that we have identified six compounds as glandular products of workers or females of six species, no volatiles were detected in cephalic extracts of workers of eight other species. As in most *Camponotus* species, males of some *Myrmecocystus* species produce volatile sex pheromones in their mandibular glands, but none are detectable as worker products (e.g., *M. christinae*, *M. wheeleri*). On the other hand, in the case of at least one species (*M. semirufus*) males and workers share two compounds (neral, geranial) but not another, methyl salicylate. Workers of one population of *M. testaceus* are noteworthy in producing, in addition to neral and geranial, the lactone mellein, a compound that is characteristic of males of many *Camponotus* species (Brand et al., 1973b). Analyses of the mandibular gland products of males of additional species will be required in order to determine how widespread the occurrence of sex-specific compounds is in the genus *Myrmecocystus*.

For the most part, the Dufour's gland constituents of workers and females are characteristic of those previously identified in species in other formicine genera, especially those of *Lasius* and *Acanthomyops* species. The alkanes, 2-alkanols, and 2-alkanones are typical products of a variety of *Lasius* (Bergström and Löfquist, 1970) and *Acanthomyops* (Regnier and Wilson, 1968) species. Hölldobler (1981) reported that the Dufour's gland secretion of *M. mimicus* contains three alkanes and two methyl ketones, compounds that we have identified in this and other *Myrmecocystus* species. Furthermore, Hölldobler demonstrated that while crushed Dufour's glands generally caused escape behavior, some workers exhibited aggressive behavior in response to the volatiles. Our results with *M. mexicanus* workers were very similar with either a crushed gland or a mixture of 2-tridecanol and 2-tridecanone.

The tridecyl esters produced by workers of *M. creightoni* and *M. colei* are the most distinctive compounds identified as Dufour's gland constituents of *Myrmecocystus* workers. Tridecyl decanoate is the only ester present in the secretion of *M. colei*, whereas five tridecyl esters fortify the exudate of *M. creightoni*. As is the case for *M. colei*, tridecyl decanoate is the major Dufour's volatile, tridecyl dodecanoate being the only other ester that is quantitatively significant. It may be chemosystematically significant that both *M. colei* and *M. creightoni* are in the small subgenus *Eremnocystus*, and it will be worthwhile to examine the Dufour's gland chemistry of other species in this taxon.

Phenol, a minor product of four species of *Myrmecocystus* is the only aromatic compound identified in the Dufour's gland secretions. This compound, which has recently been identified in a myrmicine species, *Monomorium destructor* (Blum et al., 1985), is an atypical compound in secretions that characteristically contain aliphatic constituents.

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Book Review

Introduction to Ecological Biochemistry, Third Edition. J.B. Harborne. London, Academic Press, 1988, 356 pp.

Breadth and brevity are the hallmarks of this wonderful little book. Each chapter is an engaging, skillfully crafted, self-contained discussion of a major area of contemporary research in chemical ecology. The titles of the 10 chapters are "The Plant and Its Biochemical Adaptation to the Environment," "Biochemistry of Plant Pollination," "Plant Toxins and Their Effects on Animals," "Hormonal Interactions Between Plants and Animals," "Insect Feeding Preferences," "Feeding Preferences of Vertebrates, Including Man," "The Co-evolutionary Arms Race: Plant Defense and Animal Response," "Animal Pheromones and Defense Substances," "Biochemical Interactions Between Higher Plants," and "Higher Plant-Lower Plant Interactions: Phytoalexins and Phytotoxins."

In working through this volume the reader will be treated to interesting and authoritative discussions of topics as diverse as the ecological significance of Crassulacean acid metabolism (CAM), the evolution of flower color, the chemistry and ecology of cyanogenesis, food selection by wild gorillas, the chemistry of sweetness, mammalian pheromones, allelopathy, and phytoalexins. In presenting this tremendous diversity of material, the author shifts easily between fairly detailed accounts of individual case histories, passing references to isolated but interesting facts, and lucid informative summaries of topics that have extensive bodies of literature associated with them. The chapters on pollination, pheromones, and phytoalexins are outstanding reviews of very complex subjects.

Every reader of this book will be able to think of a few topics that he or she feels ought to have been included or discussed in greater depth. For example, my list would have included a discussion of foraging energetics of bumblebees as a final section of the chapter on pollination. An account of various approaches to assess the costs of producing or detoxifying defensive chemicals would have been useful in the chapter on plant toxins. A discussion of recent work on the induction of detoxifying enzymes in insects by naturally occurring plant chemicals would have rounded out the section on the fate of plant toxins in animals. A discussion of the importance of the composition of foliage volatiles in the attraction of the Colorado potato beetle to host plants would have

added to the discussion of the role of alkaloids as feeding deterrents for this insect. A chapter on the role of microorganisms in plant-herbivore interactions would have been a good companion to the one on higher plant-lower plant interactions. I would have preferred that more space be allocated to discussions of recent work on the chemical ecology of beetle-willow interactions, pharmacophagy in Danaine and Ithomiine butterflies, iridoid glycosides and insect herbivores, and the mode of growth inhibition in insects by tannins, even if the coverage of these topics had been at the expense of space dedicated to some of the studies that so influenced this field during the 1970s.

These, however, are minor quibbles about content. The author has, in fact, exercised masterful judgment in his selection of topics. By his choice of subject matter, Prof. Harborne illustrates the broad range of questions addressed by the field of chemical ecology, introduces studies that have influenced the development of ideas in a significant way, and communicates the current excitement that pervades the field.

I am able to point to only a single topic in which the discussion has been weakened by the omission of pertinent material. In discussing possible explanations for the distribution, abundance, and diversity of plant secondary chemicals, the apparency hypothesis is presented, but there is no mention of the resource-availability hypothesis. There is also no discussion of the nutrient-balance hypothesis, of the responses of plant secondary metabolism to nutrient and light stress, or of genetically based within-species variation in secondary chemicals. As a consequence, the chapter on coevolution of plants and their herbivores is weak. It is essentially a restatement of the Ehrlich and Raven model of pairwise coevolution interpreted within the framework of the apparency hypothesis.

The value of the book as a reference work would be greatly enhanced by a better subject index, which is largely an alphabetical list of chemical names. Latin binomials for plant and animal species are separately indexed. The subject index includes very few common names of organisms and very few entries that identify processes or phenomena. For example, none of the following words or phrases are indexed: apparency, birch, deterrent, generalist, host-parasite, host selection, induced defense, induction, inhibition, inhibitor, mimicry, monarch, mutualism, parasitism, pharmacophagy, predictability, sequestration, slug, snowshoe hare, specialist, swallowtail, symbiosis, three-trophic-level interaction, trichome, variability, variation. The reader has no way of determining whether sequestration is discussed in the chapters on plant toxins, animal defenses, coevolution, or all of the above. There are no entries in the index that would direct a reader interested in the role of trichomes in plant defense to the chapters on feeding preferences and coevolution where this topic is discussed.

This deficiency in the subject index is amplified by somewhat arbitrary

placement of some topics in chapters where they do not quite seem to belong. For example, the excellent account of pheromonal interactions in pine bark beetle biology has been placed in the chapter on hormonal interactions between plants and animals rather than the one on pheromones. Since this discussion is not referenced under "pheromone" in the index, and there are no entries for "aggregation," "attraction," or "bark beetle," the only way a reader can find it is by looking up "myrcene," "pinene," or "verbenone," or by reading the entire book and remembering where it is. Other examples of questionable organization include reference to the toxic effects of many plant chemicals in the chapter on feeding attractants and deterrents, the placement of the discussion of induced plant defense in the chapter on coevolution, and a discussion of the synthesis of cardenolides by some insects in a section on pyrrolizidine alkaloids, which immediately follows a section on cardiac glycosides.

The first edition of this book was published in 1977. It had nine chapters and 243 pages. In the preface to the first edition, the author noted that the book was "based on a course taught over a number of years" and that it was "intended as an introduction to...new developments in biochemistry that have...expanded our knowledge of plant and animal ecology." A second edition appeared in 1982. The third edition has expanded to 10 chapters and 356 pages. Despite the publication of many excellent books in the area of chemical ecology during the past decade, *Introduction to Ecological Biochemistry* remains a one of a kind. It is still the only comprehensive single-author book that attempts to summarize the whole field of chemical ecology. Because of its uniqueness in this regard, it has become much more than the "simple introduction to new subject matter," it was originally designed to be. Some of us are beginning to look upon it as the book that defines what chemical ecology is. Perhaps this third edition is not yet quite chemical ecology's equivalent to Wigglesworth's *The Principles of Insect Physiology*, but at least it is close enough to suggest the analogy. Let us hope that Prof. Harborne will continue to accept the challenge of providing us with an up-to-date, expanded, and improved edition of this singularly important book every five years. In the meantime, I would encourage anybody with an interest in chemical ecology or ecological chemistry, both students who are new to the field and researchers who have been active in it for years, to buy a copy of the third edition of *Introduction to Ecological Biochemistry* and to read it from cover to cover.

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Announcements

**SEVENTH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY OF
CHEMICAL ECOLOGY (ISCE)**

The 7th meeting of the ISCE will be held jointly with the Phytochemical Society of North America (PSNA) on August 12-15, 1990 at Laval University, Ste. Foy, Province of Quebec, Canada. Among the planned symposia will be one on modern phytochemical methodology and two on chemical and ecological aspects of reproduction and defense in plants and animals. A call for submitted papers and posters will appear in January 1990.

Inquiries relative to the meeting and housing details should be addressed to:

Professor Jeremy N. McNeil
Department of Biology
Laval University
Ste. Foy, P.Q.
G1K 7P4 Canada

**5TH INTERNATIONAL WORKING CONFERENCE ON STORED-
PRODUCT PROTECTION**

- DATE AND PLACE:** September 9-14, 1990
Bordeaux, France
- ORGANIZED BY:** French National Institute of Agricultural Research
(INRA)
- PROGRAM:** Biology and physiology of insects and mites, chemical control, fumigation, pesticide resistance, control methods, sampling, rodent, birds, warm-climate protection, storage facilities, economics, modeling.
- INFORMATION:** 5th IWCSPP Conference
Secretariat, Bordeaux Congrès
33300 Bordeaux-Lac, France

REIMER-TIEMANN ADDUCTS AS POTENTIAL INSECT ANTIFEEDANT AGENTS

Reviewing the Structure–Activity Relationship Theory of the Antifeedant, Warburganal¹

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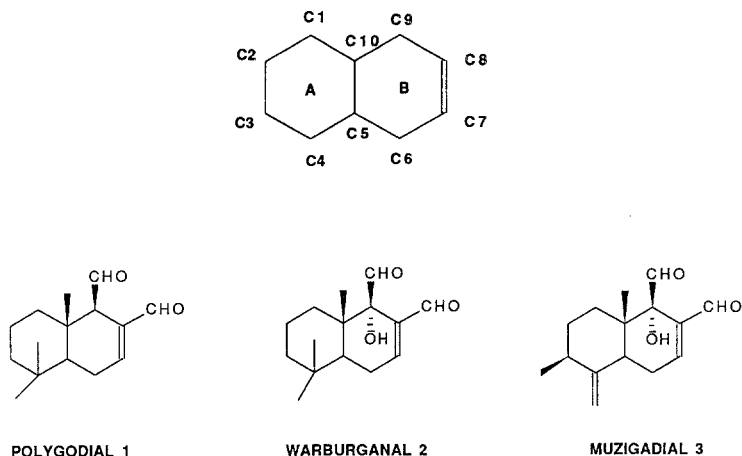
Abstract—Structure–activity relationships of naturally occurring enedials with antifeedant activity against *Spodoptera* species have been extended via the synthesis and bioassay of a series of Reimer-Tiemann adducts. The activities attributed to the different chemical structures of these and other analogs interacting with the chemoreceptor site have been observed; a three-pronged mode of substrate binding via aromatic pyrrole formation, Michael addition of free sulfhydryl moieties, and van der Waals interactions of the A ring has been postulated to account for these observations.

Key Words—Enedial, *Spodoptera*, Lepidoptera; Noctuidae, feeding deterrent, pyrrole, sulfhydryl, van der Waals interaction, Reimer-Tiemann adducts.

INTRODUCTION

Interest in the protection mechanism that plants use against pest insects prompted the isolation and identification of the insect antifeedant *Warburgia* sesquiterpene dialdehydes (Barnes and Loder, 1962; Brooks and Draffen, 1969). Some of these enedial compounds are polygodial (**1**), warburganal (**2**), and muzigadial (**3**) (Scheme 1), all of which also exhibit other biological activity including: fish

¹ Names of products in this paper are included for the benefit of the reader and do not imply endorsement or preferential treatment by USDA.



SCHEME 1.

toxicity (Asakawa et al., 1985), hot taste to humans (Govindarajan, 1979), inhibition of alcoholic fermentation (Kubo et al., 1983), and antimicrobial activity (Kubo et al., 1984). The ability of these compounds to inhibit feeding of *Spodoptera* spp. at ≥ 0.1 ppm (Kubo et al., 1977; Sodano et al., 1987) initiated interest in the syntheses of warburganal (2) (Kende and Blacklock, 1980) and cinnamodial (4) (White and Burton, 1985), as well as numerous analogs (5–12) and intermediates (Table 1).

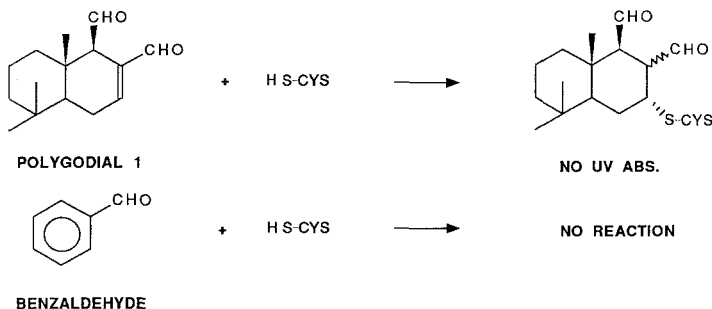
The observed biological activity led to four proposals that relate chemical structure to activity: The first suggests that when the C-8 enal moiety is allowed to react with the free sulfhydryl groups of the insect's chemoreceptor membranes, there is inhibition of stimuli transduction (Ma, 1977; Rozental et al., 1975). In support of this proposal, it was observed that α,β -unsaturated aldehydes inhibited papain (an enzyme containing 1 mol of active $-\text{SH}$) while benzaldehyde had virtually no effect. This type of reactivity is indicative of the addition of a free $-\text{SH}$ moiety to the $\beta\text{-sp}^2$ hybridized carbon atom as in a Michael addition (Scheme 2) (Kubo and Ganjian, 1981) and can be easily monitored by measuring the disappearance of the UV absorption band (λ_{max} 229 nm) of the α,β -unsaturated aldehyde. Kubo et al. (1976) found that addition of L-cysteine to a solution of polygodial at pH 7 or 9 (phosphate buffer) produced an absorbance change, as well as a decrease in the insect antifeedant activity.⁴ Several other SH-containing compounds produced similar results under these

⁴When the dialdehyde was oxidized to the diacid, the corresponding methyl diester [8] with the correct C-9 configuration showed no feeding deterrent activity at 100 ppm, indicating that the aldehydic functionalities at C-8 and C-9 are important to the biological activity (Warthen et al., 1983).

TABLE 1. ACTIVE AND INACTIVE ENEDIAL ANALOGS

ACTIVE	MODE OF ACTION ^a	INACTIVE
	A B C D E	
POLYGODIAL 1		EPIPOLYGODIAL 7
	A B C D E	
WARBURGANAL 2		ANALOG 8
	A C D E	
MUZIGADIAL 3		ANALOG 9
	A C	
CINNAMODIAL 4		ANALOG 10
	A C	
ANALOG 5		ANALOG 11
	A B C	
SACCALUTAL 6		ISOSACCALUTAL 12

^a(A) Insect antifeedant; (B) Fish toxicant; (C) Hot taste to humans; (D) Papain inhibitor; (E) Antimicrobial agent.

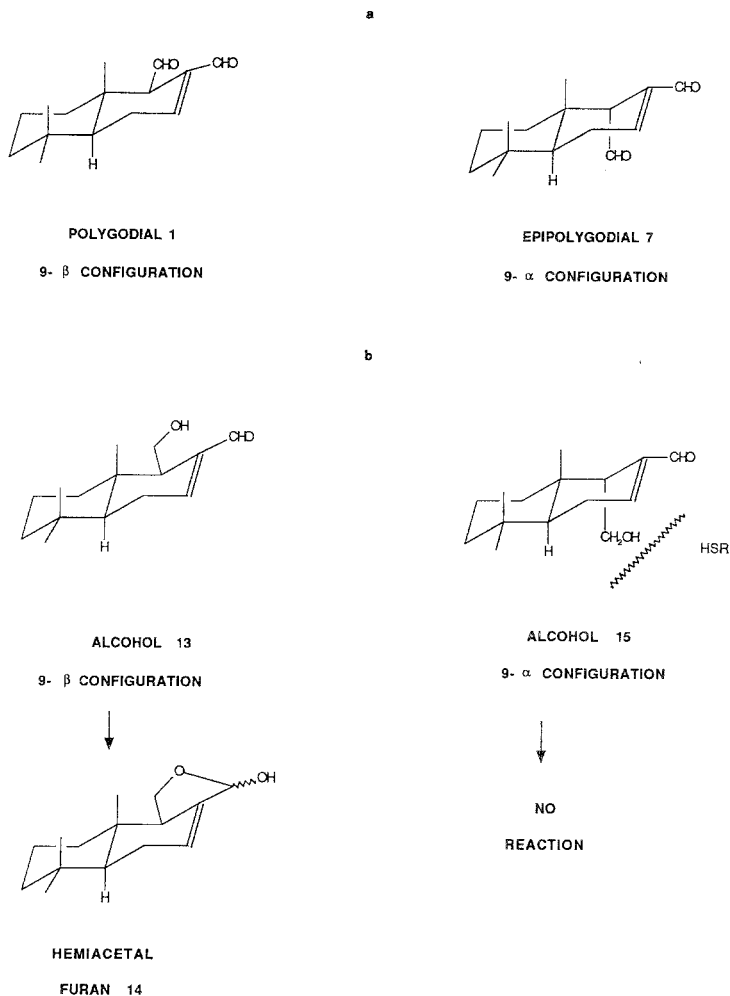


SCHEME 2.

conditions, while no absorbance change was observed when the polygodial solution was treated with a variety of amino acids that contained no $-SH$ moiety. However, because both the active polygodial (**1**) and the inactive epipolygodial (**7**) showed a similar absorbance decrease upon reaction with L-cysteine; there was speculation that the stereochemistry of polygodial was an important factor influencing biological activity since polygodial differs from epipolygodial only in the configuration of the C-9 aldehyde (Scheme 3a). The absorbance decrease produced by the reaction of polygodial and L-cysteine occurs three times faster than that produced when epipolygodial is allowed to react with L-cysteine. Clearly, the configuration of the C-9 substituent plays a critical role by interacting with approaching nucleophiles and, consequently, may be important for binding of the substrate to the receptor site.

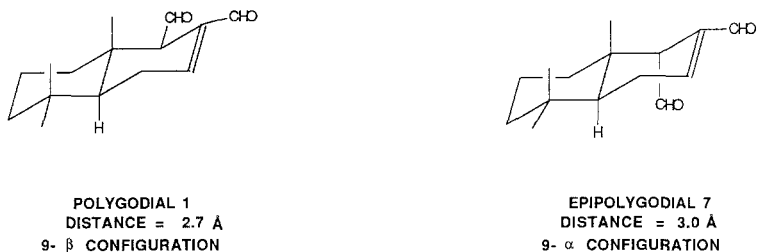
Kubo et al. (1984) tested this hypothesis by examining the interactions of the C-9 methanol analogs of polygodial and epipolygodial with L-cysteine. The 9- β alcohol (**13**) showed no UV absorbance even before the addition of L-cysteine, since an intramolecular addition of the CH_2OH hydroxyl moiety to the C-8 carbonyl had produced a hemiacetal furan (**14**). The UV absorbance (α,β -unsaturated aldehyde) of the epipolygodial analog (**15**) was virtually unchanged after the addition of L-cysteine, supporting the hypothesis that the C-9 aldehydic substituents in the α -configuration inhibit addition of the free $-SH$ moiety to the double bond of epipolygodial (Scheme 3b). However, Kubo et al. (1984) did not explain why the 9- β alcohol (**13**) formed a furan ring via intramolecular hemiacetal formation while the 9- α alcohol (**15**) did not react in this fashion.

Sodano et al. (1982) provided the second proposal to explain the biological activity of these enedials and the differences in activity between polygodial and epipolygodial. They postulated that pyrrole formation by reaction of the C-8, C-9 dialdehyde moieties with a primary amine of the receptor site was responsible for the antifeedant activity. Under biomimetic conditions, polygodial was allowed to react with a variety of primary amines, including: L-cysteine, L-lysine, L-alanine, and methylamine. In each case the amine caused a decrease



SCHEME 3.

in UV absorbance associated with the α,β -unsaturated aldehyde. Sodano et al. (1982) used Driending models to calculate the intramolecular distances between the aldehyde carbonyls of polygodial and epipolygodial, respectively (Scheme 4); they concluded that the 9- β isomer's carbonyl groups were close enough to allow for intramolecular ring closure while the 9- α isomer's carbonyls were too far apart for this to occur, thereby showing that the configuration of the C-9 aldehyde plays a critical role in the binding of the substrate to the chemoreceptor site. Additional evidence was obtained to support this hypothesis when two new

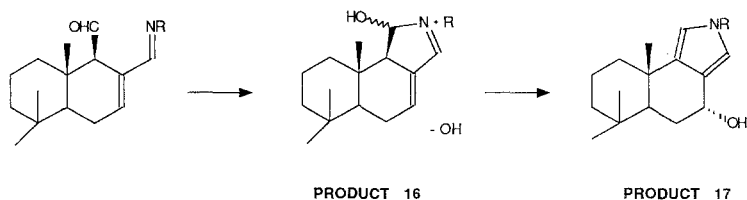


SCHEME 4.

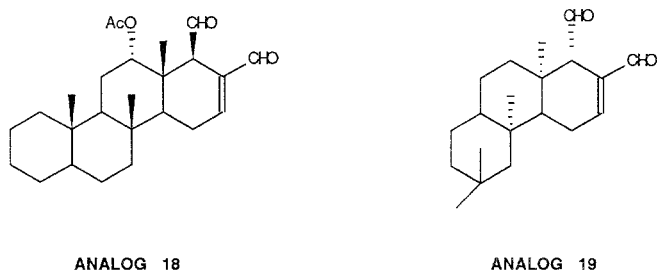
intermediates (**16** and **17**) were identified from the various reactions of primary amines on the α,β -unsaturated aldehydic carbonyl moiety of polygodial (Scheme 5) (Sodano et al., 1984). The pyrrole formation was only observed for molecules that had the appropriate stereochemical configuration (i.e., the 9- β of the *trans*-fused decalins) to allow intramolecular cyclization. Sodano et al. (1984) suggested that this information confirmed that pyrrole formation was responsible for the observed biological activity.

The third proposal relating the chemical structure of the enedials to biological activity was also made by Sodano et al. (1987), when it was observed that the steroidal analogs (**18** and **19**) of polygodial, having the identical C-9 polygodial stereochemical configuration (Scheme 6), were inactive. These authors claimed that the large steroid ring system inhibited the molecule from reaching the active site, thereby preventing any reaction with the $-\text{NH}_2$. Thus, there was a molecular size limitation even though the polygodial functionality and stereochemical backbone were present. The fourth proposal by these same authors originated because the addition of a prenyl substituent to the α -C-4 methyl group of polygodial (saccalutal, **6**) and epipolygodial (isosaccalutal, **12**) had no effect on the activity of either molecule. Thus, it seems as though a change of the alkyl substituent on C-4 of polygodial has no effect on insect antifeedant activity.

This paper reports new information relating the chemical structure of enedial analogs to insect antifeedant activity.



SCHEME 5.



SCHEME 6.

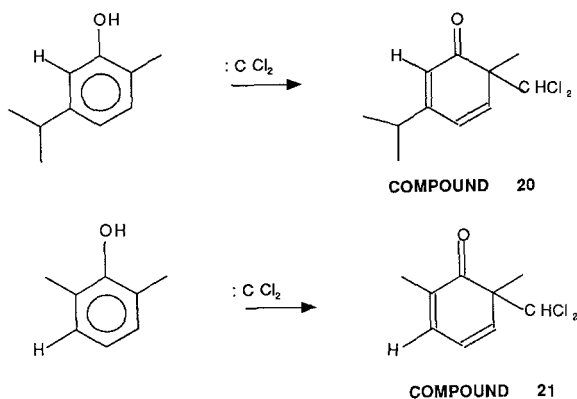
METHODS AND MATERIALS

Gas-liquid chromatography (GLC) analyses were performed on a Hewlett Packard 5830A instrument equipped with a split injector (flame ionization detector, He carrier gas). A DURAWAX-DX1 (J&W Scientific, Inc.) capillary column (0.25 mm \times 15 m) was operated at the temperature ranges indicated. Infrared (IR) spectra were obtained with a Nicolet 60SX Fourier Transform IR spectrometer using neat sample smears between KCl plates. ^1H nuclear magnetic resonance (NMR) spectra, unless otherwise stated, were obtained with a Nicolet (General Electric) QE 300 MHz instrument. All spectra were obtained using deuteriochloroform (CDCl_3) solutions with tetramethylsilane (TMS) as an internal standard. Flash chromatography by the procedure of Still (1984) was performed using Bio-Rad silicic acid-HA (-325 mesh). Analytical thin-layer chromatography (TLC) was performed on prescored silica gel GF (250- μm) plates (Analtech, Inc). Hydrogenations were performed using a Pressure Reaction Apparatus (Parr Instrument Company, Inc.). All commercially obtained chemicals were used without further purification.

Chemicals

6-Dichloromethyl-6-methyl-3-(2-propyl)-2,4-cyclohexadienone (20). The dienone (Scheme 7) was prepared in 8% yield and had physical constants identical to those first reported by Wenkert (1970), who had prepared this compound in a similar yield by refluxing a two-phase mixture containing a chloroform solution of the appropriate phenol, carvacrol, and $\sim 10\%$ aq. NaOH solution. The pure sample (flash chromatography, hexane) was subjected to GLC analysis with an oven temperature at 70°C for 1 min and then programmed at $5^\circ\text{C}/\text{min}$; 98% purity, $R_t = 17.36$ min. IR (neat) 1662 ($\text{C}=\text{O}$), 1642 cm^{-1} ($\text{C}=\text{C}$).

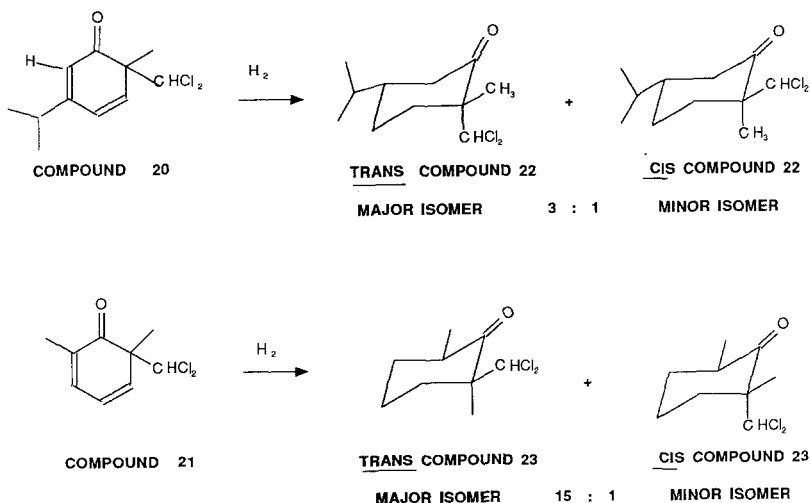
6-Dichloromethyl-2,6-dimethyl-2,4-cyclohexadienone (21). The dienone was prepared in 17% yield as described for compound **20** and was purified by flash chromatography (hexane). The pure sample was subjected to GLC analysis with an oven temperature at 90°C for 1 min and then programmed at $5^\circ\text{C}/$



SCHEME 7.

min; 99% purity, $R_f = 7.16$ min. IR (neat) 1662 (C=O), 1650 cm^{-1} (C=C); ^1H NMR (CDCl_3) δ 1.30 (s, 3H, 6- CH_3), 1.92 (bs, 3H, $\text{CH}_3\text{C}=\text{C}$), 6.09 (s, 1H, $-\text{CCl}_2\text{H}$), 6.40 (dd, 1H, $J = 7.5, 11$ Hz, C-4 H), 6.58 (d, 1H, $J = 11$ Hz, C-5 H), 6.90 (bd, 1H, $J = 7.5$ Hz, C-3 H).

cis- and *trans*-2-Dichloromethyl-2-methyl-5-(2-propyl)cyclohexanone (**22**). An ethanol solution (50 ml) of dienone **20** (2.00 g, 8.58 mmol) was hydrogenated over 10% Pd/C at 50 psi for 24 hr and filtered over Celite. The solvent was removed in vacuo; the residue was purified by flash chromatography (petroleum ether, 35–60°C) to give a 57% yield of product (1.16 g, Scheme 8). GLC



SCHEME 8.

analysis with an oven temperature at 70°C for 1 min and then programmed at 5°C/min revealed the presence of a 3:1 mixture of two diastereomers; R_f = 17.34 and 17.67 min, respectively. No attempt was made to isolate the individual isomers. Wenkert (1970) obtained a 4:1 ratio of these isomers; the isomer in the greater quantity (Scheme 8) had the isopropyl and methyl groups *trans* to each other. In our mixture the isomer in the greater quantity was identical to the *trans* isomer isolated by Wenkert (1970). IR (neat) 1716 (C=O), 762 cm^{-1} (C-Cl); [^1H]NMR (CDCl_3) δ 0.92 [m, 6H, $(\text{CH}_3)_2\text{CH}-$], 1.27 and 1.32 (s, 3H, *trans* isomer $-\text{C}-\text{CH}_3$ and *cis* isomer $-\text{C}-\text{CH}_3$, respectively), 1.32-2.49 (m, 8H), 6.19 and 6.29 (s, 1H, *cis* isomer $-\text{CCl}_2\text{H}$ and *trans* isomer $-\text{CCl}_2\text{H}$, respectively).

cis- and trans-2-dichloromethyl-2,6-dimethylcyclohexanone (23). Dienone **21** (4.83 g, 19 mmol) was hydrogenated as described for compound **22** to give a 91% yield of **23**. GLC analysis with an oven temperature of 90°C for 1 min and then programmed at 5°C/min revealed a 1:15 ratio⁵ of isomers; R_f = 7.69 and 7.73 min, respectively. IR (neat) 1702 cm^{-1} (C=O); [^1H]NMR (CDCl_3) δ 1.05 (d, 3H, $J = 6.0$ Hz, $\text{CH}_3\text{CH}-$), 1.37 (s, 3H, $-\text{C}-\text{CH}_3$), 1.30-2.20 (m, 6H, $-\text{C}-(\text{CH}_2)_3-\text{CH}-$), 2.56 (m, 1H, $\text{CH}_3\text{CHCH}_2-$), 6.20 ppm (s, 1H, $-\text{CCl}_2\text{H}$).

Bioassay

Fall armyworm larvae, *Spodoptera frugiperda* (J.E. Smith), from our stock culture, reared on an artificial diet (Redfern and Raulston, 1970), were used for antifeedant tests. The samples were dissolved at an appropriate concentration in a 50:50 acetone-dimethyl sulfoxide solvent mixture; the resultant solution was added to a hot diet and mixed by using a 1/2-pint (720 ml) jelly jar fitted with an Osterizer blender cutter head. After the treated diet was blended for approximately 1 min, it was poured into 1-oz clear plastic cups (~8 g/cup) and allowed to cool to room temperature. Control diets were only treated with the neat solvent. One first-instar larva (newly emerged) was placed in each cup (10 cups for each sample concentration). The cups were then capped with pre-punched lids and placed in a holding room at $27^\circ \pm 1^\circ\text{C}$ with $50 \pm 5\%$ relative humidity. The larvae were observed for mortality, and the live larvae weighed on the sixth or seventh day after introduction into the cups. The sum of weights for the 10 repetitions per sample, as well as the control, were then divided by

⁵The isomerization of the 15:1 mixture was accomplished by the addition of lithium diisopropylamide to a THF solution (-78°C) of **23**, stirring for 2 hr as the temperature rose to 26°C , and then acidifying with 10% (v/v) aq. HCl. GLC revealed the presence of a 50:50 mixture. Repetitive flash chromatography (petroleum ether, $35-60^\circ\text{C}$) gave a fraction in which the new isomer predominated in a 60:40 ratio. IR (neat) 1702 cm^{-1} (C=O); [^1H]NMR (CDCl_3) δ 1.05 (d, 3H, $J = 6.0$ Hz, CH_3CH), 1.27 (s, 3H, $2-\text{CH}_3$), 1.32-2.19 (m, 5H), 2.30 (ddd, 1H, $J = 3.9, 6.6, 14$ Hz; C-4 H_{ax}), 2.59 (m, 1H, $\text{CH}_3\text{CHCH}_2-$), 6.40 (s, 1H, $-\text{CCl}_2\text{H}$).

the number of live larvae weighed in order to obtain an average larval weight for each sample. The ratio of these average larval weights (treated/control) was then used to assess the antifeedant activity of the various samples (Table 2).

RESULTS AND DISCUSSION

The work of Kubo and Sodano has been very thorough. However, there are still several questions to be addressed that relate the structures of the enedials to their antifeedant activity, namely: (1) are both the C-8 and C-9 aldehydic oxidation states necessary for antifeedant activity at low sample concentrations (≤ 10 ppm); (2) if pyrrole formation is the most important reaction for activity, does the $-SH$ moiety react in conjunction with the NH_2 to enhance the activity; (3) why does the addition of the $\alpha-OH$ at C-9 of warburganal enhance the antifeedant activity compared to polygodial (no $\alpha-OH$, Scheme 1); (4) if steric bulk inhibits the approach of the substrate to the receptor site, what effect will a truncated natural product analog have; and (5) what effect do the alkyl substituents have on the antifeedant activity?

The first conclusion that can be drawn from the data in Table 1 is that the C-8 and C-9 carbon atoms must have their substituents in the aldehydic oxidation state in order to demonstrate potent antifeedant activity. This is especially apparent when comparing the structural differences of polygodial (**1**) and the diester analog (**8**) (Scheme 9). Both molecules would be expected to form a cyclic intermediate, since both corresponding β -C-9 carbonyl moieties should be in close enough proximity to the C-8 carbonyl to allow the intramolecular cyclization (see above). Once cyclization has occurred in both systems, only the intermediate arising from the dialdehyde can proceed to form the aromatic pyrrole (**24**). At this point it becomes necessary to interject our viewpoint that the sulfhydryl attack on the β -carbon of the enal, as proposed by Kubo and

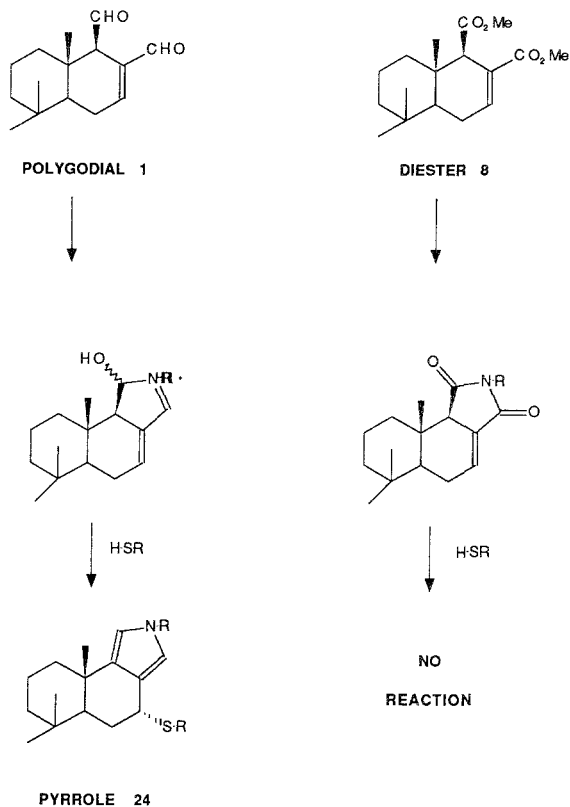
TABLE 2. ANTIFEEDANT ACTIVITY OF SYNTHETIC COMPOUNDS 20-23^a

Sample	Conc. (ppm)	Mortality	% wt. of control
Compound 20	1000	1 of 10	103
Compound 21	1000	0 of 10	21
Compound 22 ^b	1000	1 of 10	99
Compound 23 ^c	1000	1 of 10	28
Control	1000	1 of 10	100

^aNo antifeedant activity was observed for any sample at 100 ppm. Percent weight was calculated as described in Methods and Materials.

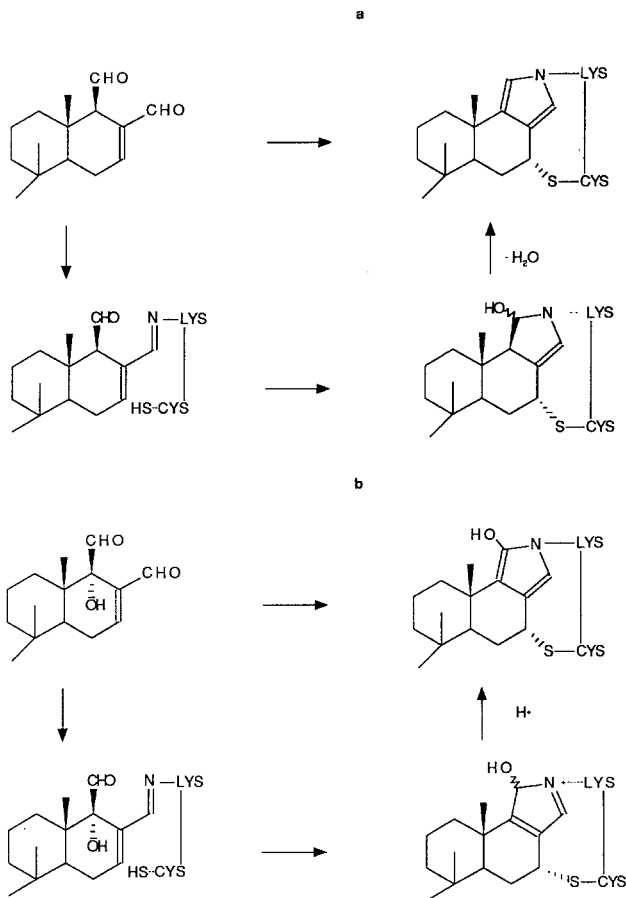
^b1:3 mixture of *cis* and *trans* isomers.

^c1:15 mixture of *cis* and *trans* isomers.



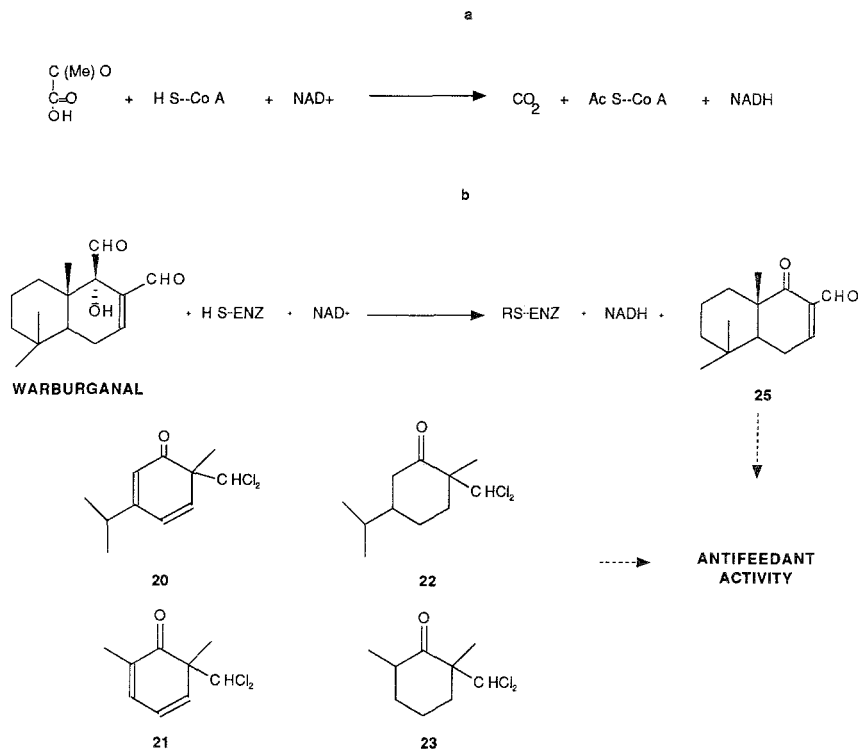
SCHEME 9.

Ganjian (1981), and the pyrrole formation by NH_2 attack on the aldehyde carbonyls, as proposed by Sodano et al. (1982), are not necessarily independent reactions. One can hypothesize that both the L-lysine $-\text{NH}_2$ and the L-cysteine $-\text{SH}$ moieties of the chemoreceptor site complex with the substrate in accordance with both the Sodano and Kubo mechanisms in order to produce the biological activity (Scheme 10a). Initial imine formation at the C-8 carbonyl followed by β -addition of the SH and subsequent ring closure and pyrrole aromatization would produce a product similar to compound **24**. This mechanism could explain why warburganal is a more potent antifeedant than the simple polygodial. For example, if the α -C-9 hydroxide were displaced by an $\text{S}_{\text{N}}2'$ attack of the SH moiety on the β -carbon of the enal, the resultant alkene would force the C-8 and C-9 substituents into an even closer proximity; thereby enhancing the pyrrole formation (Scheme 10b).



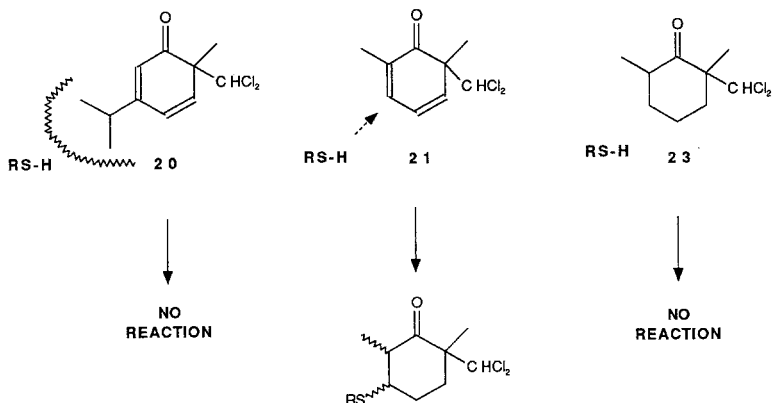
SCHEME 10.

There are other mechanisms by which SH moieties are known to react in vivo. One of the more common reactions is the oxidative decarboxylation reaction of the acid dehydrogenase (Scheme 11a) (Lehninger, 1975). This type of reaction could occur with compounds like warburganal (Scheme 11b) to generate an intermediate (**25**), which would then induce the antifeedant activity; our synthetic compounds, **20** and **21**, would be expected to react in a similar fashion with the sulfhydryl moiety via Michael addition to the alkene. The synthetic compounds **20–23** would also be expected to form an imine via coupling of the substrate's dichloromethyl moiety (carbon in the aldehydic oxidation state) and amine groups of the active site in a manner identical to that expected of the aldehyde moiety of the warburganal metabolite, **25**. Compounds **20–23** would



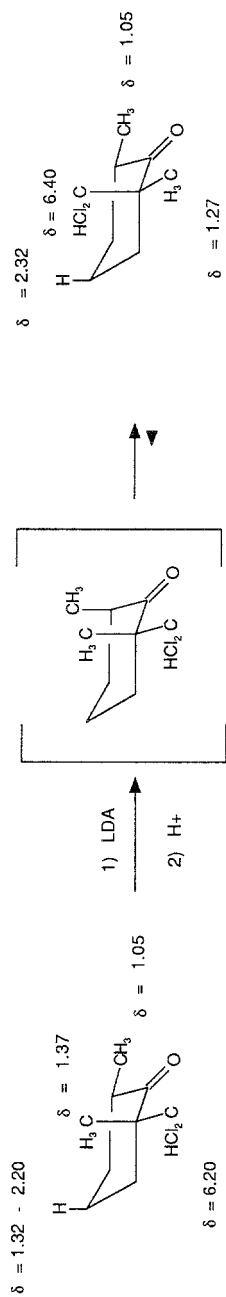
SCHEME 11.

therefore be expected to be as active as warburganal since the chemical reactivity is similar to that proposed for the intermediate (**25**) and other quinones that are known to elicit antifeedant activity (Rozental et al., 1975). Unfortunately, all the synthetic compounds were inactive at 100 ppm, and this mechanistic pathway was therefore discarded in favor of the one shown in Schemes 10a and 10b. Obviously, these cyclohexane molecules are not inactive at the 100-ppm level because their steric bulk prevents binding with the chemoreceptor site, as in the case of the steroidal analogs of polygodial. Since the synthetic samples are not sterically prohibited from reaching the receptor site, it must be the inability of compounds **20–23** to form the pyrrole ring that accounts for their inactivity at low sample concentration. This observation supports the idea that the C-8 and C-9 aldehydes are necessary for antifeedant activity at lower concentrations. However, the 1000-ppm test results indicate that some dramatic differences exist between the inactive compounds, **20** and **22**, and the active compounds, **21** and **23**.



SCHEME 12.

Compounds **20** and **21** differ only in the alkyl substitution pattern (Scheme 12). Our reasoning used to explain the difference in activity was that the two methyl groups of the C-3 isopropyl substituent on compound **20** were sterically blocking the SH from adding to the β -carbon of the α,β -unsaturated ketone in the same manner that the 9- α CH_2OH prevented sulfhydryl coupling in alcohol **15** (Scheme 3b). This could not explain why the saturated analog **23** was nearly as active as compound **21** at 1000 ppm (72% and 79% weight reduction, respectively) since **23** had no alkene with which the SH could react. In hindsight, it was no surprise that compounds **20** and **22** were inactive, since it had previously been demonstrated that the alkyl substitution changes that produced saccalutal (**6**) from polygodial (**1**) and isosaccalutal (**12**) from epipolygodial (**7**) had had virtually no effect on the activity of the compounds. Apparently then, it is the C-2 methyl group of compound **21** and the corresponding C-6 methyl of compound **23** that was responsible for the difference in activity between these two active compounds and their inactive counterparts, **20** and **22**. We noted that the bridge-head methyl group had been reported to exist exclusively in the axial position in all of the naturally occurring antifeedant compounds **1-4**, and **6** (Table 1), while the C-2 methyl group of compound **21** and the C-6 methyl group of compound **23** were both equatorial (the C-2 methyl of compound **21** lies in the plane of the cyclohexadiene ring and can therefore be formally considered equatorial, Schemes 7 and 8). Epimerization of compound **23** produced only equatorial methyl groups, as determined by proton NMR analysis (Scheme 13). Alkyl groups have been known to effectively bind to chemoreceptor sites via dispersion and van der Waals forces that require tight fits to the active site since the magnitude of these attractive forces decreases rapidly with distance (Liljefors and Thelin, 1985). There is, therefore, precedence to expect that an



TRANS 23

CIS 23

SCHEME 13.

appropriately positioned alkyl substituent would enhance the binding ability of the substrate to the active site once pyrrole formation had occurred. The alkyl substitution patterns of our active molecules correspond to the C-1 methylene in the A ring of the naturally occurring enedial antifeedants. Our findings suggest that a three-pronged binding between the substrate and the receptor site, which involves pyrrole formation, Michael addition of the sulfhydryl moiety, and van der Waals interactions of the A ring, occurs to produce the antifeedant activity.

CONCLUSION

Data are provided to show that only compounds having a 1,4-dialdehyde moiety will allow the formation of an aromatic pyrrole, which is necessary in order to observe insect antifeedant activity at low sample concentrations. The reaction of the —SH moiety in conjunction with this pyrrole formation was postulated when it had been observed that the C-8 and C-9 diester analog of polygodial had no antifeedant activity at 100 ppm. Although this compound could form a pyrrole-like product in vivo (the mode of action proposed for polygodial), further reaction with the free sulfhydryl of the chemoreceptor site would be impossible. Differences in the antifeedant activities of compounds **20–23** support the idea that the activities of the naturally occurring enedial antifeedants, produced by pyrrole formation and sulfhydryl attack, may be enhanced by the van der Waals interactions of the A ring with the receptor site, although these weak interactions are obviously not a prerequisite for activity.

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RESPONSES FROM SENSILLA ON ANTENNAE OF MALE *Heliothis zea* TO ITS MAJOR PHEROMONE COMPONENT AND TWO ANALOGS¹

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Abstract—For some species, chemical analogs have been identified that can substitute for the major pheromone component and reduce pheromone-trap captures in atmospheric permeation experiments. The ability to substitute these analogs for the major pheromone component in field tests raises the question: Do the same set of olfactory receptor neurons on the insects' antennae respond to both the major component and the effective structural analogs? To investigate this question, extracellular responses were recorded from single sensilla on the antennae of male *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) to stimulation with increasing doses of (Z)-11-hexadecenal, the major pheromone component, (Z)-9-tetradecen-1-ol formate, and (Z)-1,12-heptadecadiene. Both (Z)-9-tetradecen-1-ol formate and (Z)-1,12-heptadecadiene can substitute for (Z)-11-hexadecenal in trap disruption experiments and affect the sexual behavior of males, although neither has been identified in the volatiles released by calling females. All three compounds elicited responses from the same class of pheromone-sensitive receptor neurons over a range of stimulus concentrations. At equivalent stimulus concentrations, however, (Z)-11-hexadecenal elicited about a 10-fold greater response than (Z)-1,12-heptadecadiene. (Z)-9-Tetradecen-1-ol formate elicited a response slightly higher than, but not significantly different from, the response to (Z)-1,12-heptadecadiene.

¹Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the U.S. Department of Agriculture or the State of Florida.

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Key Words—Pheromone, disruption, atmospheric permeation, neurophysiology, *Heliothis zea* (Boddie), Noctuidae, Lepidoptera.

INTRODUCTION

Recently, much effort has been devoted to investigating the potential use of pheromones and other semiochemicals in the control of insect pest species (Mitchell, 1981). One control strategy involves the disruption of normal chemical communication between males and females by releasing large quantities of the major pheromone component into the atmosphere surrounding an agricultural crop or commodity (Shorey et al., 1974; Mitchell, 1975; Sanders, 1981). Ideally, this atmospheric permeation with pheromone prevents many males from being able to locate females, thereby drastically reducing insect mating and subsequent egg production. The efficiency of this approach is severely comprised in those species whose major pheromone component is unstable in storage or in the field. This has prompted evaluation of closely related analogs of major pheromone components with the view toward finding more stable mimics (Beevor and Campion, 1979; Carlson and McLaughlin, 1982a,b; Silk et al., 1985; Silk and Kuenen, 1986; Curtis et al., 1987).

Heliothis zea (Boddie) (Lepidoptera: Noctuidae), the corn earworm moth, is one insect where communication disruption using both pheromone components and analogs has been demonstrated (Mitchell, 1975; Mitchell et al., 1976; McLaughlin et al., 1981; Tingle and Mitchell, 1982; Mitchell and McLaughlin, 1982). Female *H. zea* produce a complex blend of pheromones that attract conspecific males for mating. The major component of their blend, (*Z*)-11-hexadecenal (AL), constitutes approximately 92% of the volatiles released by calling females (Klun et al., 1979). Mitchell et al. (1975) have demonstrated that in addition to disruption using AL, a structural analog, (*Z*)-9-tetradecen-1-ol formate (FO), can also disrupt this insect's communication. Carlson and McLaughlin (1982a,b) have shown a similar disruption ability with *H. zea* using another structural analog, (*Z*)-1,12-heptadecadiene (OL). Neither FO nor OL have been identified in gland extracts or volatiles released from this species.

The apparent ability of these analogs to substitute for the major pheromone component in disruption experiments raises an interesting question concerning the detection of these semiochemicals by the insect's peripheral sensory system. In most lepidopteran systems studied to date, receptor neurons sensitive to pheromone components show a high degree of selectivity for their target compounds. Consequently, one might question whether the effective mimics would stimulate the same or a different population of olfactory receptor neurons. Several researchers (Mitchell et al., 1975; Priesner, 1979) have suggested that such

chemical mimics compete for activation sites on the same set of olfactory receptor neurons, which are responsive to the major component of the pheromone. To investigate the specificity of pheromone-sensitive neurons, we recorded responses using standard extracellular recording techniques from olfactory receptor neurons contained within sensilla on the antenna of male *H. zea* in response to increasing doses of AL, FO, and OL. We investigated those sexually dimorphic sensilla that are oriented in rows along the proximal 40 flagellar subsegments on the male antenna.

METHODS AND MATERIALS

Insects and Chemicals. Male *H. zea* were taken as pupae from colonies maintained at this laboratory. Insects were held at 75–85% relative humidity and 24–26°C under a 14:10 hr light–dark regimen. Neurophysiological recordings were conducted 24–72 hr following adult emergence. Chemical samples used in this study were provided by Dr. J. H. Tumlinson (AL and FO) and Dr. D. A. Carlson (OL). (Z)-11-Hexadecenal was analyzed by GLC on a OV-1, 36-m capillary column and found to be 99.3% pure. (Z)-1,12-Heptadecadiene was analyzed on a DB-1, 15-m capillary column, and no impurities, such as AL, were detected. (Z)-9-Tetradecen-1-ol formate was analyzed on 3% OV-1 on 100–120 mesh Gas Chrom-Q (1.8 m × 2 mm ID glass column) and found to >99% pure.

Recordings. Insects were secured for single sensillum recordings with low-melting-point wax and their antennae positioned to allow access with microelectrodes. The microelectrodes, electrolytically sharpened tungsten wire (tip diameters approx. 1 μm), were held and positioned under a compound microscope (E. Leitz, Inc., Rockleigh, New Jersey; 600×) with micromanipulators (E. Leitz). The indifferent electrode was inserted into the lumen of a flagellar subsegment on the distal one third of the antenna, and the recording electrode was positioned at the base of an individual sensillum trichodeum on a more proximal subsegment. Penetration of the sensillum cuticle with the recording electrode was accomplished by gently tapping the manipulator along the long axis of the electrode. The signals from the microelectrodes were amplified by a Grass P-18 preamplifier and sent concurrently to a Tektronix 5113 storage oscilloscope for observation and to a Digital PDP-11/23 minicomputer for data acquisition and analysis (Mankin et al., 1987).

Following penetration of the sensillum by the recording electrode, the preparation was allowed to stabilize for 10–15 min prior to the first stimulus presentation. During this period and throughout the recording session, purified carrier air (1000 ml/min) passed over the preparation to isolate the sensillum

from potentially contaminated room air. The spontaneous activity of each neuron in the sensillum was measured before stimulation and was monitored at intervals during the recording session to assess the stability of the preparation.

Stimulation. The methods of dispenser preparation and stimulus delivery are described elsewhere (Mayer, 1973; Grant et al., 1989). Briefly, desired quantities of the chemical component, diluted in 0.5 ml of hexane, were distributed over the inner surfaces of the cylindrical glass dispensers. Following the evaporation of the hexane solvent, the ends of the assembly were attached by ground-glass joints to the stimulus delivery system. Control dispensers contained only 0.5 ml of hexane solvent. Delivery of the stimulus was accomplished by combining a stimulus airstream (200 ml/min), which passed through the pheromone-dosed dispenser, with a purified carrier airstream (1000 ml/min). The mixture of these two streams subsequently passed over the preparation. Initiation and termination of the stimulus period was accomplished by computer-controlled activation and deactivation of a solenoid valve attached to the stimulus airstream (Mankin et al., 1987; Grant et al., 1989). In all cases, 10 sec of neural activity were recorded. This 10 sec was composed of three periods: prestimulus (0–3.0 sec), stimulus (3.0–6.0 sec), and poststimulus (6.0–10.0 s). Responses are expressed as the mean frequency of impulses generated during the stimulus period minus the mean frequency of impulses generated during the prestimulus period.

Dose-Response Relationships. To establish mean dose-response relationships, increasing doses of AL were presented to at least 21 preparations. To establish the mean dose response relationships for FO and OL, each of 10 preparations was stimulated a single time at each dose. Each preparation was exposed to graded doses of only one stimulus compound. In all cases, the lowest dose stimulus was presented first, followed by stimuli of increasing dosages. Interstimulus intervals ranged from 4 to 10 min depending on the magnitude of the previous response. Control stimuli were presented approximately every five stimulations. The actual concentrations that stimulated the antenna were estimated from emission rate calibrations (Mayer et al., 1987).

Scanning Electron Microscopy. To determine the distribution of the sexually dimorphic sensilla on the male antenna, three antennae were examined using scanning electron microscopy. Micrographs were taken of every tenth subsegment beginning at the tenth flagellar subsegment from the pedicel. Prior to sputter coating with 60:40 gold-palladium, portions of the antenna were washed in spectrophotometric grade hexane, allowed to air dry, and grounded to the grid supports with silver paint. Coated specimens were immediately examined with a Hitachi 500 STEM microscope at 30 kV accelerating voltage and magnifications to 25,000 \times . Micrographs were recorded with Polaroid Type 55 P/N film.

RESULTS

Distribution of Sensilla. In agreement with a previous description of the antennal morphology of *H. zea* (Callahan, 1969), we found sexually dimorphic sensilla trichodea oriented in rows along the lateral surfaces of the proximal 40–50 flagellar subsegment (Figure 1A,B). These sensilla were absent from the distal 20–30 subsegments (Figure 1C) and appeared to be typical lepidopterous sensilla trichodea (e.g. Zacharuk, 1986), with their surfaces annulated and sparsely pitted by pores (Figure 1D). From our micrographs, we estimate that there are approximately 2200 of this type of sensillum on each male antenna.

Response Characteristics. Extracellular recordings from *H. zea* indicate that each sensilla trichodeum is innervated by at least two spontaneously active receptor neurons. The action potentials range in amplitude from 50 to 400 μV and have typical durations of approximately 2.5 msec. These impulses can be reliably discriminated from each other by their amplitudes and waveforms. The receptor neuron producing the larger impulse is designated as A and the neuron producing the smaller as B. In several of the preparations, very small impulses ($< 30 \mu\text{V}$) were observed that could not be reliably discriminated from the noise.

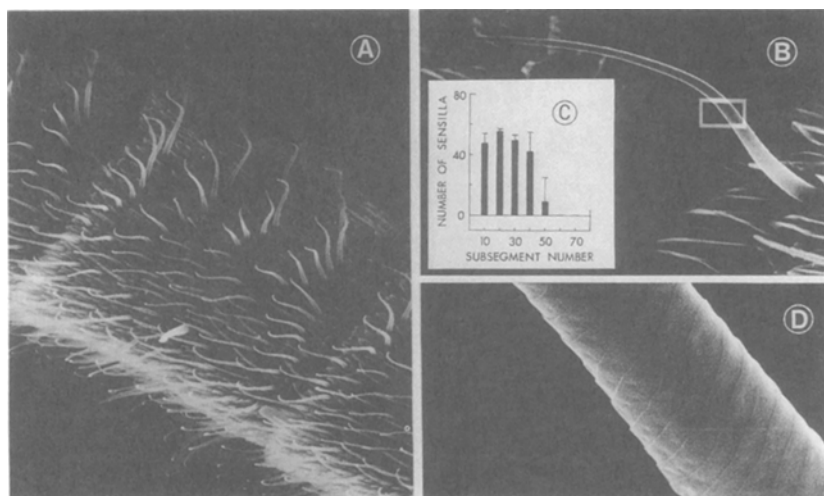


FIG. 1. Scanning electron micrographs of the antenna of a male *H. zea*. (A) A flagellar subsegment showing the rows of long, sexually dimorphic sensilla trichodea. (B) A single s. trichodeum from the distal row located on one of the proximal subsegments. (C) A histogram of the mean ($\pm\text{SD}$) number of sensilla in rows on every tenth subsegment ($N = 3$) from the antennal base. (D) A higher magnification of the area enclosed by the square in Figure 1B illustrates the sensillar surface and pores.

For this reason, their responses were excluded from further analysis in this report.

Dose-Response Relationships. Responses from the A neuron were consistently observed to stimulation with each of the three compounds tested; AL, FO, and OL. No responses were observed from the B neuron to stimulation by any of these compounds at any of the doses tested. The observation that one class of receptor neuron responds to each of these three stimuli does not preclude the possible existence of other classes of receptor neurons in other morphological types of sensilla that may well have more pronounced differences in response selectivity with respect to these three compounds.

In all cases, responses from the A neuron were such that an increase in stimulus dosages led to an increase in the frequency of impulse production during the stimulus period. Mean responses of the A neurons to stimulations with different doses of AL, FO, and OL are shown in Figure 2A. In addition to similarities in the mean discharge frequencies, similarities also were observed in the averaged temporal discharge pattern in response to stimulation with equal doses of each compound (Figure 2B). With all these compounds, the initial part of the response was enhanced as stimulus intensity was increased. Since the OL was synthesized via a Wittig reaction with AL as the starting material (Carlson and McLaughlin, 1982b), the possibility exists that trace amounts of AL could be in the sample, yet go undetected by conventional GC analysis. However, since samples of the olefin produced via the synthetic route described above were shown to be >99%, the responses seen in Figure 2A cannot be attributed solely to the presence of trace AL contaminants in the OL sample.

DISCUSSION

The neurophysiological data presented here indicate that there is a class of receptor neurons on the antenna of male *H. zea* that responds to stimulation with the major pheromone component and to stimulation with two other analogs. The mean dose-response curves for the three compounds are quite similar (Figure 2A). However, the similarity in dose-response curves may be misleading, since these chemicals differ by over half an order of magnitude in vapor pressure (Carlson and McLaughlin, 1982b) and consequently may differ in the rate at which they are released from the surface of the dispenser. Therefore, comparisons among the responses from equivalently dosed stimuli should be evaluated in terms of the concentration differences among the compounds and not solely on the amount of material loaded into a stimulus cartridge.

To make comparisons on the basis of concentration, emission rates were calibrated from the stimulus dispensers for a series of long-chain hydrocarbons (Mayer et al., 1987), including the three compounds evaluated in this study.

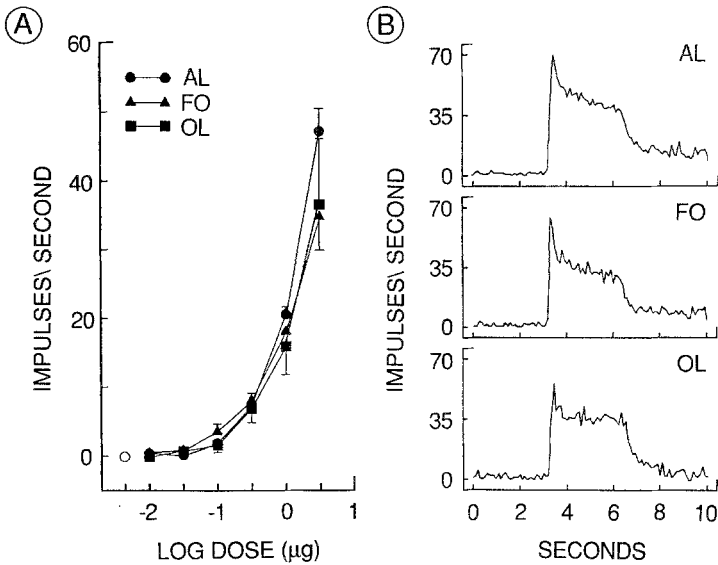


FIG. 2. (A) Mean (\pm SEM) dose-response relationships from the A neuron of *s. trichodea* on the antenna of male *H. zea* to stimulation with increasing doses of (Z)-11-hexadecenal (closed circles), (Z)-9-tetradecen-1-ol formate (closed triangles), and (Z)-1,12-heptadecadiene (closed squares). Standard error bars have been omitted from some mean values to enhance clarity of the figure. Doses on the *x* axis equal the amount of material loaded into the dispensers and not the amount reaching the preparation. The mean response to the hexane control stimulation is indicated by the open circle. (B) Averaged event-time histograms illustrating responses from the A neuron to stimulation with equal doses (3.16 μ g) of (Z)-11-hexadecenal ($N = 29$); (Z)-9-tetradecen-1-ol formate ($N = 11$); and (Z)-1,12-heptadecadiene ($N = 10$). Responses are averaged in 100-msec intervals. The stimulus period began at 3.0 sec and ended at 6.0 sec.

From the measured concentration emitted from the dispenser at known doses, we calculated regression equations for each material, relating the amount of material loaded into the dispenser (dose in micrograms) to the amount of pheromone emitted from the dispenser outlet. Concentration in micromoles/per cubic centimeter can be calculated from emission rate, given the total rate of flow of stimulus-laden air over the antenna (concentration = emission rate/1200 cm^3/min). Due to the extreme sensitivity of olfactory receptor neurons to stimulations with semiochemicals, the lowest stimulus dose for which we could reliably quantify the emission rate was equal to the highest dose tested in these electrophysiological studies. It was necessary to estimate concentrations for lower doses (those effective in the electrophysiological studies) by extrapolating from the dose emission functions in Mayer et al. (1987). The resulting concentration

estimates were then used to replot (Figure 3) the data shown in Figure 2. When replotted, there is a larger quantitative difference among the mean stimulus-response curves elicited by the three compounds. (*Z*)-11-Hexadecenal is a more effective stimulant than either (*Z*)-9-tetradecen-1-ol formate or (*Z*)-1,12-heptadecadiene. It should be noted, however, that these differences in sensitivity are relatively small when compared to the very large differences expected when pheromone-sensitive receptor neurons are stimulated with other nonpheromone compounds.

These neurophysiological responses are difficult to interpret unambiguously because the processes involved in disruption of insect chemical communication by atmospheric permeation with the major pheromone component are not well understood. For example, modification of the insect's sensory system by either adaptation of the peripheral receptor neurons or habituation of the central nervous system has been proposed to explain elements of the disruption process (Bartell, 1982). Alternatively, persistent activity in these pheromone-

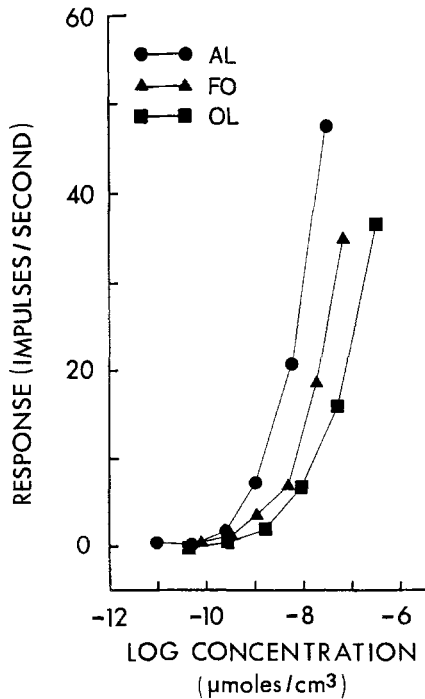


FIG. 3. Mean concentration vs. response relationships from the A neuron to stimulation with increasing concentrations of (*Z*)-11-hexadecenal (closed circles); (*Z*)-9-tetradecen-1-ol formate (closed triangles), and (*Z*)-1,12-heptadecadiene (closed squares).

sensitive receptor neurons may act to confuse or modify the normal behavioral response of the male and render him unable to orient to a calling female. A widely accepted theory proposes that individual trails of pheromone emanating from calling females are camouflaged by the large synthetic release of pheromone in disruption experiments (Cardé, 1981). Whatever the mechanism, we assume that in order for disruption to occur, the major pheromone component must interact in some fashion with the highly sensitive pheromone receptor neurons in the trichoid sensilla. Additionally, the present data suggest that the olefin and the formate analogs may achieve their disruptive effects via interaction with the same population of olfactory receptor neurons that are sensitive to the major pheromone component.

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WING FANNING AS A MEASURE OF PHEROMONE RESPONSE IN THE MALE PINK BOLLWORM, *Pectinophora gossypiella* (LEPIDOPTERA: GELECHIIDAE)

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Abstract—A still-air, wing fanning bioassay measured male pheromone response in the pink bollworm moth, *Pectinophora gossypiella* (Saunders). As pheromone concentration was increased, the duration of wing fanning increased while its latency decreased. Response duration was longest for the natural pheromone blend [44:56 (*Z,E*)- and (*Z,Z*)-7,11-hexadecadienyl acetates], although responses to 38% and 50% *Z,E* blends were not significantly briefer. Preexposure to pheromone had no effect on wing fanning in males retested after 24 hr. Wing fanning was highly correlated with upwind flight and other responses observed in a wind tunnel. Wing fanning duration provides a continuous measure of pheromone response and allows a large number of males to be tested in a short time; these two characteristics of the wing-fanning assay are helpful for genetic analyses of male response.

Key Words—Pink bollworm, *Pectinophora gossypiella*, Lepidoptera, Gelechiidae, sex pheromone, (*Z,E*)-7,11-hexadecadienyl acetate, (*Z,Z*)-7,11-hexadecadienyl acetate, bioassay.

INTRODUCTION

The evolution of pheromone communication systems depends on their genetic architecture and the selective forces acting upon them. We have examined the genetic basis of pheromone communication in the pink bollworm moth, *Pectinophora gossypiella* (Saunders) (Collins and Cardé, 1985, 1989a,b, 1990; Col-

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lins et al., 1990). The pheromone of this moth is a female-produced, two-component blend of (*Z,E*)- and (*Z,Z*)-7,11-hexadecadienyl acetates (*ZE* and *ZZ*, respectively) (Hummel et al., 1973; Bierl et al., 1974). The mean blend found in pheromone gland extracts is 44% *ZE* (Collins and Cardé, 1985). This pheromone is used by males to locate females and also induces courtship (Colwell et al., 1978; Guerra, 1968).

In this study, we describe a still-air, wing-fanning bioassay and the effect of various pheromone dose-blend combinations on male pheromone response. Wing fanning in the pink bollworm is a component of courtship behavior (Colwell et al., 1978; Guerra, 1968). This bioassay was developed for studies of the genetic bases of male response.

Criteria for a diagnostic bioassay are that the behavior(s) included are well correlated with the success of mate finding and that they provide a continuous (graded) measure of response. We examined the relationship of wing fanning to other pheromone-induced behaviors that are expressed in a wind tunnel. To gauge the relative responses of individual males to two ratios of pheromone components, males must be tested twice. However, male response is inhibited by preexposure to pheromone 1 hr before testing (Collins, 1988). The effect of initial pheromone exposure on wing fanning responses in bioassays conducted 24 hr after preexposure is documented in this paper.

METHODS AND MATERIALS

Insects and Test Conditions. Moths were obtained as pupae from the USDA APHIS Pink Bollworm Rearing Facility in Phoenix, Arizona, where they have been reared in the laboratory for more than 50 generations. Male pupae were placed in 100- × 15-mm glass screw-cap test tubes upon receipt. Moths used in the wind-tunnel study were from a laboratory colony that was started from pupae obtained from the USDA rearing facility (Collins and Cardé, 1985). The wheat germ-soybean flour diet and rearing methods were modified from those of F.D. Stuart (personal communication). Three larvae were reared with ca. 5 g of diet medium in 21-ml clear plastic cups covered with opaque plastic lids. Pupae were removed from the diet after 17 days, sexed, and held singly in 85- × 15-mm glass test tubes with cork stoppers. All pupae were checked daily for adult emergence.

All life stages were held at $26 \pm 1^\circ\text{C}$ and 15:9 hr light-dark. Inadvertent exposure to pheromone prior to testing was avoided by holding the males in a controlled environment chamber isolated from female moths.

Males were tested during the second and third scotophases postemergence. All tests were conducted between 3.5 and 0.5 hr before the end of the scotophase, corresponding to the flight activity period in the field when males are

most responsive to pheromone (Graham et al., 1964; Van Steenwyk et al., 1978). All tests were conducted at $26 \pm 1^\circ\text{C}$ with a light intensity of 1.8 lux, which is within the optimal response range (Farkas et al., 1974). Males were acclimated to these conditions for at least 15 min prior to testing.

Pheromone Stimulus Preparation. For all pheromone blends tested, $10 \mu\text{g}/\mu\text{l}$ concentrations in *n*-hexane were prepared gravimetrically from synthetic pheromone (Farchan Chemical Co.). Lower concentrations were made by serial dilutions in decade steps to $10^{-4} \text{ ng}/\mu\text{l}$. Stimuli were applied in $10 \mu\text{l}$ of solvent to 0.5-cm-wide strips of Whatman No. 1 filter paper, and the solvent was allowed to evaporate. Stimuli were kept in separate 30-ml plastic cups with plastic lids until used. Control stimuli in all experiments consisted of $10 \mu\text{l}$ of *n*-hexane on filter paper.

Bioassay Tubes. The effects of preexposure and of various dose-blend combinations were tested using 10-cm-long \times 2.5-cm-diam glass bioassay tubes with 1-cm-long central transverse slits. Relatively small tubes were used to reduce variation in pheromone concentration that could result from movement of the moth during the bioassay, to facilitate observations under low light intensity, and to permit the storage and handling of a large number of males without having to remove residual pheromone from the bioassay chambers between tests.

On the day after emergence, males were transferred to individual bioassay tubes and the open ends and transverse opening sealed with parafilm. During testing, the transverse opening was uncovered to allow insertion of the pheromone stimulus. Inadvertent exposure to extraneous pheromone during the bioassay was obviated by directing air filtered with activated charcoal and glass wool over the bioassay tube and exhausting this air from the bioassay room.

Effect of Dose-Blend Combination. Treatments consisted of all combinations of five stimulus dosages (10^{-3} to 10 ng of pheromone in decade steps) and seven component blends (10, 25, 38, 44, 50, 65, and 90% ZE). A modified randomized complete block design was used. Blocks consisted of three to five observations for each of the 36 treatments (35 dose-blend combinations and the control). Blocks were completed in two to three consecutive days of testing.

Each male was observed for ca. 2 min prior to testing. Males that wing fanned spontaneously were discarded. The parafilm covering the transverse slit in the bioassay tube was then removed and the male was observed for an additional 10–15 sec and discarded if wing fanning was observed. Fewer than 1% of males were discarded before testing. The filter paper strip impregnated with the appropriate blend and concentration of synthetic pheromone was then inserted. Each male was observed for 2 min. Latency of reponse (time elapsed from the introduction of the stimulus to the initiation of wing fanning) and total duration of wing fanning were recorded. For males that did not wing fan, latency was defined as 120 sec. Males were bioassayed once.

Effect of Preexposure. To examine the effect of preexposure to pheromone

on subsequent response, individual males were bioassayed twice. Males were first exposed during the second scotophase following adult emergence. The stimuli used consisted of 10 ng of pheromone in blends of 10, 44, or 90% ZE. After testing, the bioassay tubes were resealed with parafilm and returned to the controlled environment chamber. Twenty-four hours later these males were exposed to stimuli containing 1 ng of pheromone in blends of 10, 25, 44, 65, or 90% ZE. The combination of blends used for each male was selected randomly.

The effect on wing fanning of bioassay-tube contamination during preexposure was checked. Stimuli with 10 ng of 44% ZE were placed in 10 empty tubes for 2 min. No wing fanning was observed when males were placed in these tubes 1 hr later.

Wind-Tunnel Bioassays. The relationships between wing fanning and other pheromone-mediated responses were examined by observing the response of males to different pheromone blends in a small wind tunnel patterned after that of Cardé and Hagaman (1979), but lacking a visual pattern on the tunnel floor. The Plexiglas wind tunnel was 81 cm long with crosswind dimensions of 28 × 28 cm. Wind velocity was 0.30 ± 0.05 m/sec. Light was provided by a DC-main fluorescent light shielded with a red filter eliminating wavelengths below 680 nm. Light intensity at the middle of the tunnel was 1.8 lux.

Stimuli used in the wind-tunnel bioassays consisted of 10 ng of pheromone containing 25, 44, or 65% ZE. This concentration elicited response levels that allowed differentiation among the 44% ZE and the off-blends (25 and 65% ZE). The filter-paper stimuli were attached to the end of 12-cm-long wires inserted into cork stoppers. After solvent evaporation, these stimuli were placed in 2.5- × 20-cm test tubes until used. New stimuli were prepared immediately before the start of each day's bioassays. For each test, a stimulus was placed in the center of the wind tunnel 10 cm from the upwind end. A male was then released 50 cm downwind from the pheromone source. A test tube containing a male was opened and placed on a 10-cm-high metal platform 50 cm downwind with the open end facing upwind. The occurrences of five behaviors were recorded: (1) wing fanning before taking flight; (2) zigzag, upwind flight in which the male approached within 10 cm of the source; (3) landing on the pheromone source; (4) wing fanning after landing at the source; and (5) wing fanning latency. Wing fanning that lasts but a few seconds either may not be evoked by pheromone or may be caused by very low levels of pheromone contamination (see Results). Therefore, only wing-fanning bouts lasting more than 5 sec were tabulated. Each male was observed until it wing fanned after landing at the source or until 7 min had elapsed.

Statistical Analysis. The significance of differences in the duration of wing fanning in still-air bioassays for various dose-blend combinations and for multiple pheromone exposures were evaluated with the STP method of nonpara-

metric multiple comparisons (Sokal and Rohlf, 1981, p. 438). The significance of differences in the percent of males that wing fanned to various pheromone blends was determined using Friedman's method (Sokal and Rohlf, 1981, p. 446), treating observations made at different concentrations as randomized blocks. A chi-square test was used to compare response incidences among dose-blend combinations. The effect of dose-blend combinations on latency of wing fanning among all males tested was assessed with the STP method; the latency for males that did not wing fan was assumed to be 120 sec (the length of the observation period). Specific distributions were compared using the Mann-Whitney U test (Sokal and Rohlf, 1981, p. 433).

Percentages of response for the three blends tested in the wind tunnel were compared using a chi-square test. The significance of wing-fanning latency was evaluated by the Kruskal-Wallis test (Sokal and Rohlf, 1981, p. 430).

RESULTS AND DISCUSSION

Dose-Blend Combinations. The cumulative durations of wing fanning in response to various dose-blend combinations ranged from 0.1 ± 0.3 to 77.1 ± 37.4 sec (mean \pm SD) (Figure 1). Response was longest at the highest concentration (10 ng) for all blends and generally higher for blends closer to the natural (44% ZE) blend. No significant differences were found among the responses to any blend for the lowest pheromone concentration tested (10^{-3} ng), but these were not different from the control (0.7 ± 2.1 sec) (STP; $P > 0.05$).

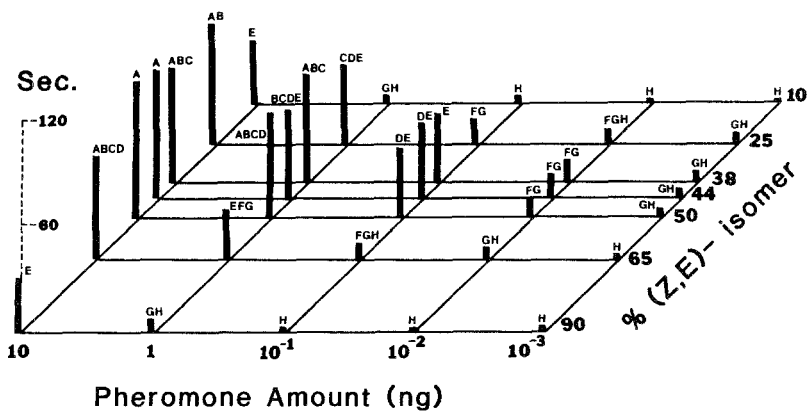


FIG. 1. Mean duration of wing fanning as affected by pheromone blend and concentration; $N = 40$ per dose-blend combination. Means not followed by a common letter are significantly different, STP multiple comparison test; $P < 0.01$.

The durations of male wing fanning in response to blends containing 38, 44, and 50% ZE were not significantly different at any concentration tested (STP; $P > 0.05$). These percentages are, respectively, the mean blend emitted by calling females (Haynes et al., 1984), the mean blend found in pheromone gland extracts (Collins and Cardé, 1985), and the blend used commercially to disrupt mating in field populations (Doane and Brooks, 1981). This demonstrates a relatively broad response spectrum for wing-fanning duration and is consistent with patterns of trap catch (Flint et al., 1979) and wind-tunnel response (Linn and Roelofs, 1985).

The percentage of males that wing fanned in response to various dose-blend combinations ranged from 5.0% to 95.0% (Figure 2). Pheromone blend had a significant effect on the incidence of response (Friedman's test, $\chi^2 = 73.5$; $P < 0.01$). The overall patterns observed for the incidence and duration of wing fanning were similar. However, the effects of blend and concentration on wing fanning duration were more pronounced. Although 17.5% of males wing fanned to filter paper treated with solvent, the mean duration of their wing fanning was only 0.7 sec. This suggests that duration is more discriminating than response incidence in separating responses to different blends and concentrations.

Response latencies were longer for lower doses and for extreme blends, and thus, inversely related to wing-fanning duration (Figure 3). No significant differences were found among the latencies for the lowest pheromone concentration tested (10^{-3} ng) and the control (107.9 ± 31.2 sec) (STP; $P > 0.05$). Latencies for blends containing 38, 44, and 50% of the ZE isomer were not

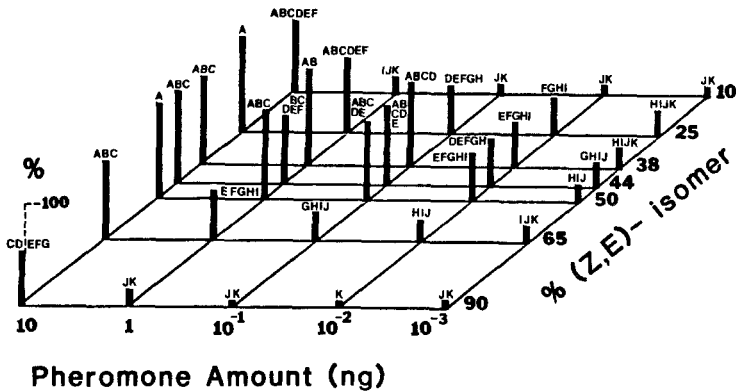


FIG. 2. Percentage of males wing fanning as affected by pheromone blend and concentration; $N = 40$ per dose-blend combination. Means not followed by a common letter are significantly different, χ^2 test; $P < 0.01$.

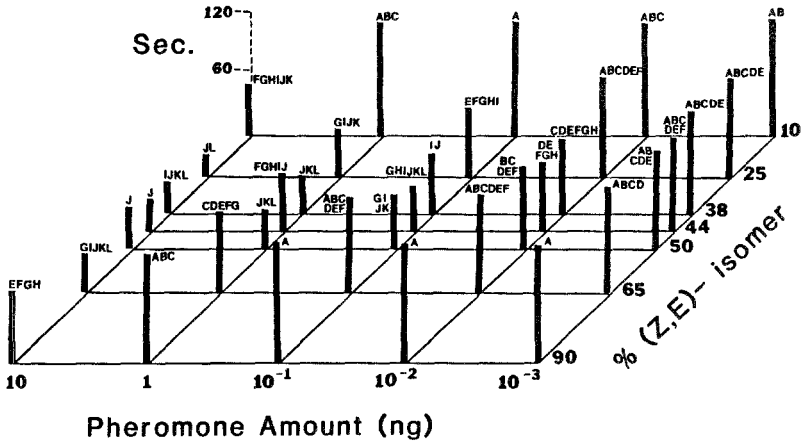


FIG. 3. Mean latency of wing fanning as affected by pheromone blend and concentration; $N = 40$ per dose-blend combination. Means not followed by a common letter are significantly different, STP multiple comparison test; $P < 0.01$.

significantly different at any of the concentrations tested (STP; $P > 0.05$), reflecting the same broad response spectrum as wing-fanning duration.

Although qualitatively similar, the relationships between latency and stimulus parameters were not as pronounced as for wing fanning duration (Figure 1), and latency was more variable than duration (coefficients of variability among males that wing fanned = 70.0% and 140.3%, respectively). These results suggest that the duration of wing fanning is a more reliable measure of male response than latency.

The relationship between concentration and response in the pink bollworm appears to be direct: increased pheromone doses evoke increased duration of wing fanning. The relationship between pheromone ratio and the intensity of wing fanning also appears to be straightforward: the incidence of response is higher and response duration is elevated for ratios approaching the natural blend.

Effect of Preexposure. Preexposure did not affect the duration of wing fanning in bioassays conducted 24 hr later, as the same response patterns were observed in both tests (Table 1). For each blend used in the second exposure, the duration of wing fanning was not significantly affected by the stimulus to which the male was preexposed (STP multiple comparison test; $P > 0.05$). No pattern is evident in these data to suggest that initial exposure alters subsequent response in a predictable manner.

Wind-Tunnel Bioassays. One or more overt response behaviors were observed for 20.0, 72.5, and 27.5% of the males tested in the wind tunnel using blends with 25, 44, and 65% ZE, respectively ($N = 40$ per blend) (Table 2).

TABLE 1. MEAN (\pm SD) WING-FANNING DURATION FOR SEQUENTIAL EXPOSURES TO PHEROMONE SEPARATED BY 24 HR^a

First Exposure ^b	Second exposure ^c				
	10% ZE	25% ZE	44% ZE	65% ZE	90% ZE
Control					
28.c (8.6)	20.1cde (31.6)	54.5abc (39.1)	72.6a (39.5)	35.7abcde (39.7)	15.3e (34.9)
10% ZE					
50.3b (42.3)	14.2e (26.3)	52.3abc (38.6)	58.5ab (40.9)	17.7de (32.1)	10.8e (20.2)
44% ZE					
82.7a (29.5)	9.7e (21.2)	48.7abcd (36.1)	52.1abcd (37.7)	22.3cde (34.1)	27.0bcde (40.1)
90% ZE					
51.8b (44.0)	4.8e (9.6)	39.9abcde (35.7)	51.0abcd (39.2)	29.7bcde (39.4)	6.9e (18.3)

^a Amounts of pheromone used in the first and second exposures were 10 and 1 ng, respectively.

^b For responses observed during first exposure (column 1), means not followed by a common letter are significantly different, STP multiple comparison test; $P < 0.01$.

^c For responses observed during second exposure, means not followed by a common letter are significantly different, STP multiple comparison test; $P < 0.01$.

TABLE 2. FREQUENCY OF RESPONSE SEQUENCES TO THREE PHEROMONE BLENDS IN A WIND TUNNEL^a

PW	Behavioral sequence ^b			25% ZE	44% ZE	65% ZE	Total
	UF	LS	WF				
*				1	2		3
	*			1		1	2
	*	*		1		1	2
	*	*	*	3	16	9	28
*	*	*	*	2	11	1	14
	No response			32	11	28	71

^a Stimuli contained 10 ng of pheromone.

^b Behaviors: PW, preflight wing fanning; UF, upwind flight; LS, landing on the pheromone source; and WF, wing fanning after landing.

The percentage of males that wing fanned after landing at the 44% *ZE* source was 67.5% and was significantly different from the 12.5 and 25.0% for the 25 and 65% *ZE* blends ($\chi^2 = 22.97$ and 12.87 , respectively, $P < 0.01$). The percentages of males that wing fanned after landing on the 25 and 65% *ZE* stimuli were not significantly different ($\chi^2 = 1.31$, $P > 0.05$). The relationship between pheromone blend and response incidence in the wind tunnel is consistent with relationships observed in the still-air bioassays (Figures 1 and 2).

Among males that flew upwind, 91% (42/46) landed at the source and wing fanned after landing. All males that landed at the source wing fanned. Wing fanning was always accompanied by clasper extension (as described by Colwell et al., 1978). Among 49 responding males, the sequence of flight, landing, and wing fanning was observed for all but seven individuals.

In the wind tunnel, male pink bollworms usually take flight abruptly and without discernible preflight behavior. Wing-fanning behavior in our wind tunnel was qualitatively indistinguishable from wing fanning observed in the glass bioassay tubes. Preflight wing-fanning bouts appeared identical to bouts that took place after landing on the pheromone source and are assumed to be courtship related. Preflight wing fanning was observed for 17 males; 14 of these flew upwind, landed, and wing fanned after landing. No males exposed to the control responded.

Among males that wing fanned after landing on the source, the mean (\pm SD) wing-fanning latencies were 1.95 ± 2.17 , 2.19 ± 1.91 , and 2.86 ± 2.87 min for the 25, 44, and 65% *ZE* blends, respectively. These latencies were not significantly different (Kruskal-Wallis test, $H = 2.20$; $P > 0.05$).

Our wind-tunnel bioassays suggest that the incidence and duration of wing fanning of confined males are well correlated with upwind flight and landing near the pheromone source. A similar correlation has been demonstrated for the Oriental fruit moth, *Grapholita molesta* (Busck) (Baker and Cardé, 1979): once a male wing fans, he is very likely to complete the entire response sequence. However, this relationship is not universal. For example, in *Ostrinia nubilalis* (Hübner) the characteristics of wing fanning of males confined in a 2.5-cm-diam \times 90-cm-long glass tube with a 1.2 m/sec air flow (Webster and Cardé, 1984) did not reflect the discrimination found in a wind-tunnel assay (Glover et al., 1987). Similarly, the duration of preflight wing fanning and dosage are unrelated for the gypsy moth, *Lymantria dispar* (L.) (Cardé and Hagaman, 1979; Hagaman and Cardé, 1984). However, in this species wing fanning prior to flight is evidently involved in thoracic temperature regulation (Cardé and Hagaman, 1983).

Conclusions. The wing-fanning bioassay used in this study appears to provide a reliable indicator of the effects of blend and concentration on pheromone-mediated attraction and courtship behavior in *P. gossypiella*. It also provides a continuous measure of response intensity useful in genetic analyses. Traits with discrete phenotypes (e.g., upwind flight) must be based on assumed (but not

directly observable) underlying continuous variables (Falconer, 1981). The wing-fanning bioassay is simple and allows many moths to be processed in a short interval. To evaluate the response of males to a spectrum of pheromone stimuli, individual males must be tested repeatedly. The preexposure experiment indicated that males may be retested on consecutive days without discernible alterations in the pattern of response on the second day. Examples of the application of this assay in measurements of the heritability of male response are given by Collins and Cardé (1989a) and in artificial selection by Collins and Cardé (1990).

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HERITABLE VARIATION IN PHEROMONE RESPONSE OF THE PINK BOLLWORM, *Pectinophora gossypiella* (LEPIDOPTERA: GELECHIIDAE)

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Abstract—Heritability of variation in male pheromone response by pink bollworm moths, *Pectinophora gossypiella* (Saunders), was examined using a still-air, wing-fanning bioassay. Heritability (\pm SE) of overall responsiveness, as measured by the mean duration of wing fanning to the blend of pheromone components produced by females [44:56 ratio of (*Z*, *E*)- to (*Z*, *Z*)-7,11-hexadecadienyl acetate], was 0.385 ± 0.095 . Heritabilities of wing-fanning duration to blends with 25 and 65% *Z*, *E* isomer were 0.377 ± 0.113 and -0.145 ± 0.103 , respectively. These findings indicate an asymmetry in the genetic component of variation in response to pheromone blends with high and low proportions of the *Z*, *E* isomer. An index of response specificity for individual males was developed based on the response to an off-blend (either 25 or 65% *Z*, *E* isomer) relative to the response to the 44% *Z*, *E* blend. Heritabilities of response specificity were 0.117 ± 0.059 and -0.043 ± 0.067 for the 25 and 65% *Z*, *E* blends, respectively.

Key Words—Pink bollworm, *Pectinophora gossypiella*, Lepidoptera, Gelechiidae, sex pheromone, genetics, heritability, pheromone response, (*Z*, *E*)-hexadecadienyl acetate, (*Z*, *Z*)-7,11-hexadecadienyl acetate.

INTRODUCTION

The genetic bases of pheromonal communication in the pink bollworm moth, *Pectinophora gossypiella* (Saunders), are of particular interest because synthetic pheromone is used commercially to disrupt mating (Doane and Brooks, 1981;

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Baker et al., 1989), and this application creates the potential for the evolution of new pheromone strains that are resistant to such disruption. The sex pheromone of the female pink bollworm is a blend of the (*Z*, *E*)- and (*Z*, *Z*)-isomers of 7,11-hexadecadienyl acetate (*ZE* and *ZZ*, respectively) (Hummel et al., 1973; Bierl et al., 1974). Females produce a blend containing a mean (\pm SD) of $44.2 \pm 2.3\%$ *ZE* (Collins and Cardé, 1985). In contrast to the narrow variance signal produced by females (Collins and Cardé, 1985; Haynes et al., 1984), pink bollworm males are attracted to a relatively broad range of pheromone blends (Flint et al., 1979; Haynes et al., 1984).

Since efficient communication depends on the coordination of signaling and reception, evolutionary change in pheromone-mediated systems depends upon the nature and extent of heritable variation in pheromone production and response. Heritable variation in the blend and amount of pheromone produced by female *P. gossypiella* has been documented (Collins and Cardé, 1985, 1989a; Collins et al., 1990). The current study is an analogous investigation of heritability in male pheromone response using a wing-fanning bioassay. Wing-fanning duration increases with concentration and is greater for blends more closely approximating the blend normally produced by females. Wing fanning is well correlated with other pheromone response behaviors, including upwind flight (Collins and Cardé, 1989b).

This paper examines variation in wing fanning and estimates heritabilities for overall responsiveness to various blends, the specificity of response to particular blends, response latency, and response thresholds in a laboratory population. Heritability measures the fraction of phenotypic variance that is available to selection and is a function of the genes influencing the character, environmental conditions, and genotypic frequencies in the population (Falconer, 1981). Heritability also measures the degree of resemblance between relatives and can be used to predict the rate of change in average phenotype in response to selection pressure in the field or in the laboratory.

METHODS AND MATERIALS

Moths. Pupae were obtained from USDA, APHIS in Phoenix, Arizona. These pupae were sexed and the females discarded. Inadvertent exposure to pheromone prior to testing was avoided by placing male pupae individually in 85- × 15-mm test tubes with screw-caps or cork stoppers and keeping them in a controlled environment chamber at 27°C and 15:9 hr light-dark, isolated from sources of natural and synthetic pheromone. Pupae were checked daily for adult emergence. On the day after emergence, each male was transferred to a 10-cm-long × 2.5-cm-diam glass bioassay tube with a 1-cm-long centrally

located transverse opening (Collins and Cardé, 1989b). The ends and transverse opening were sealed with parafilm.

Stimulus Preparation. Pheromone stimuli used in the bioassays consisted of various combinations of concentrations and blends of the *ZE* and *ZZ* isomers. For all blends, 10 $\mu\text{g}/\mu\text{l}$ concentrations in *n*-hexane were prepared gravimetrically from synthetic compounds (Farchan Chemical Co.). Lower concentrations were made by serial dilutions in decade steps to 10^{-4} $\text{ng}/\mu\text{l}$. Each stimulus was applied in 10 μl of solvent to a strip of filter paper. Solvent was allowed to evaporate, and the stimuli were kept in separate 30-ml clear plastic pill cups with plastic lids. New stimuli were prepared immediately before the start of each day's bioassays.

Bioassay. A still-air, wing-fanning bioassay (Collins and Cardé, 1989b) was used to measure male response. Males were first tested during the second or third scotophase. Each male was observed for ca. 2 min prior to bioassay. If spontaneous wing fanning was observed, the male was discarded. The parafilm covering the transverse opening in the bioassay tube was then removed and the male observed for an additional 10–15 sec and discarded if wing fanning was observed. Each male was then bioassayed by inserting the filter paper stimulus with the appropriate blend and concentration of pheromone into the bioassay tube through the transverse opening. Males were observed for 2 min using 10^{-1} ng of pheromone. In a previous study, only 1.8% of males wing fanned for more than 110 sec, and none wing fanned for more than 117.8 sec using this dose for any blend tested in this study (Collins and Cardé, 1989b). Latency (the time elapsed from the introduction of the stimulus to the start of wing fanning) and total duration of wing fanning were recorded using two stop watches. Bioassays were conducted during the last 4 hr of the scotophase when males are most responsive (Graham et al., 1964; Van Steenwyk et al., 1978) at 27°C and 1.8 lux.

Males were bioassayed twice, first with a 44% *ZE* blend (the blend normally produced by females), and then with an off-blend containing either 25 or 65% *ZE*. The two tests for each male were conducted on consecutive days. When pheromone exposures are separated by 24 hr, the initial exposure does not alter the subsequent response in a consistent or systematic manner (Collins and Cardé, 1989b).

Responsiveness. Two aspects of male response were examined: overall responsiveness and response specificity. The general level of responsiveness was measured in two ways: (1) the latency and duration of wing fanning to the 44% *ZE* blend, and (2) the sum of the responses to both stimuli (the 44% *ZE* blend and the off-blend).

Response Specificity. A specificity index (*S*) was calculated by comparing relative responses to the normal (44% *ZE*) and off-blends (25 or 65% *ZE*) using

a modification of the Shannon-Weaver index. This index equals 0.0 for males that respond only to the off-blend, 1.0 for males that respond only to the normal blend, and 0.5 for males that do not differentiate between the blends (Figure 1). Its construction is described in Appendix A.

Heritability Estimation. Heritabilities of characters associated with pheromone response behaviors were estimated by parent-offspring regressions. Males in the parental generation were bioassayed and then mated with virgin females selected randomly from our laboratory colony. Male-female pairs were placed in 7.5- × 7.5- × 2.8-cm clear plastic oviposition cages. Sucrose solution was available to the adults in 1.9-ml vials with cotton wicks on the bottom of each cage. Paper oviposition substrates were changed daily. To segregate the progeny of each male-female pair, oviposition substrates were sealed in 15- × 15- × 2-cm plastic bags until the larvae hatched.

Newly hatched larvae were placed individually in 21-ml clear plastic cups covered with plastic lids. Each cup contained ca. 5 g of wheat germ-soybean flour diet (Collins and Cardé, 1985). Pupae were removed after 17 days, sexed, and placed singly in 85- × 15-ml glass test tubes with cork stoppers. All life stages were kept at 27°C and 16:8 hr light-dark photoperiod.

Male offspring from these matings were bioassayed as before. Approximately five male offspring per family were tested with the same stimuli and order of presentation used to test their fathers. Heritabilities of response param-

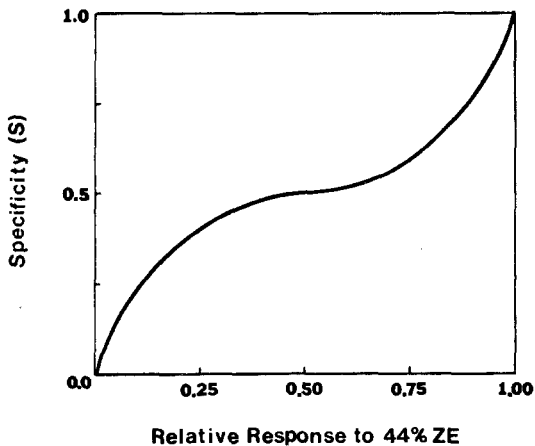


FIG. 1. Response specificity (S) as a function of the relative response to the normal (44% ZE) blend, $R_n [= D_n / (D_n + D_o)]$, where D_n and D_o are the wing-fanning durations for the normal and off-blends, respectively].

eters were estimated by the regression of parent (father) on mean offspring values. The number of male offspring available for testing varied among families. Thus, regression coefficients were estimated by weighting families according to the number of offspring measured (Falconer, 1981, p. 167).

The duration of wing fanning was not normally distributed because many males did not wing fan. Thus, nonparametric tests were used in intergenerational comparisons. The Mann-Whitney U statistic was calculated for each comparison, and a value, t_s , was computed based on this statistic (Sokal and Rohlf, 1981, p. 435) and used to test the significance of observed differences. The percentages of males that wing fanned to different pheromone stimuli within generations were compared using a χ^2 test.

Threshold. Response threshold was measured by the latency of wing fanning of males exposed to a series of increasing pheromone concentrations. Males were obtained as pupae and reared as before. Each male was tested on two consecutive days. During each test, the male was exposed to a series of stimulus concentrations. Test series consisted of six pheromone stimuli in 10 μ l of *n*-hexane, increasing by decade steps from 10^{-3} ng to 10^2 ng of total pheromone (*ZE* plus *ZZ* components). All stimuli in series A contained 25% *ZE* isomer, and series B contained stimuli with 65% *ZE*. Males that were exposed to series A on the second day postemergence were tested against series B on the third day, and vice versa.

Males were exposed for 30 sec to each stimulus, from the lowest to the highest concentrations. Each stimulus was kept in the bioassay tube until the next stimulus was introduced. Tests were terminated upon initiation of wing fanning. Prior to each stimulus series, the parafilm was removed from the transverse slit in the bioassay tube and the male observed for 30 sec. Males that spontaneously wing fanned during this period were discarded. Latency was the time elapsed from the introduction of the first stimulus until wing fanning. The primary advantage of this approach to estimating response latency is that nearly all males respond.

After testing, males were paired with virgin females selected randomly from a laboratory colony, and the offspring of these pairings were reared as before. Male offspring were tested using the same stimuli series used to test their male parents.

Latency of response to all blends was estimated by the sum of the latencies observed during exposure to each of the two stimulus series. The degree of symmetry associated with responses to blends with high and low percentages of the *ZE* isomer was measured by the difference between the latencies observed for the two stimulus series. Heritabilities of these latency measures were estimated by a weighted regression of mean offspring on paternal values.

RESULTS

Overall Responsiveness. The duration of wing fanning in a 2-min observation period to 10^{-1} ng of pheromone containing 44% ZE isomer was 46.10 ± 30.97 sec (mean \pm SD). In subsequent exposures to 25 and 65% ZE stimuli, mean responses were 19.45 ± 27.47 and 10.18 ± 25.38 sec, respectively. These findings are consistent with those of a previous study (Collins and Cardé, 1989b).

These males were used to produce a second generation for the estimation of heritabilities. Mean response durations for blends with 25, 44 and 65% ZE isomer among F_1 males were 16.18 ± 24.53 , 41.79 ± 34.07 , and 8.46 ± 21.10 sec, respectively. The mean responses in the parental and F_1 generations were not significantly different for each blend tested (Mann Whitney U test; $t_s = 0.84, 1.60, \text{ and } 0.94$ for the 25, 44, and 65% ZE blends, respectively; $P > 0.05$). Therefore, random drift or unidentified selective forces did not appear to affect gene frequencies at loci influencing wing fanning.

A higher percentage of males wing fanned to the 25% ZE blend than to the 65% ZE blend in both generations. Of the males tested in the parental generation, 61.70 and 36.71% responded to the 25 and 65% ZE blends, respectively (significantly different, $\chi^2 = 9.75, N = 173; P < 0.01$). Similarly, 55.17 and 30.75% responded to the 25 and 65% ZE blends, respectively, in the F_1 generation (significantly different, $\chi^2 = 44.407, N = 754; P < 0.01$).

Males that were more responsive to the 44% ZE blend also tended to be more responsive to blends with 25 or 65% ZE. The durations of wing fanning in the two tests were significantly correlated in both the parental and F_1 generations (Table 1). These data suggest that some males were more responsive than others over a relatively wide range of component blends. Thus, differences

TABLE 1. CORRELATIONS BETWEEN DURATION OF WING FANNING TO INITIAL STIMULUS WITH 44% ZE ISOMER AND WING-FANNING DURATION IN SECOND BIOASSAY WITH 25 OR 65% ZE BLEND

Generation	Second stimulus	Correlation coefficient	N	F
Parents	25% ZE	0.422	94	20.25 ^a
	65% ZE	0.394	79	14.44 ^a
Offspring	25% ZE	0.198	406	16.81 ^a
	65% ZE	0.298	348	33.64 ^a

^aSignificantly different; $p < 0.01$.

among males did not appear to be the result of different response thresholds for specific blends.

Significant correlations were found between the wing-fanning duration observed in the first and second bioassays for each male within both the parental and F_1 generations. (Table 1; coefficients transformed by the inverse hyperbolic tangent; $z = 2.15$, $P < 0.05$; Sokal and Rohlf, 1981, p. 583). The reason for the difference between generations is not known, and there were no other significant differences among correlation coefficients.

Heritability of Responsiveness. Estimated heritability (\pm SE) of responsiveness, measured by wing-fanning duration to a 44% ZE blend, was 0.385 ± 0.095 (statistically significant, $t = 4.052$, $N = 173$; $P < 0.01$) (Figure 2b). Thus, 38.5% of variance in the observed response to the 44% ZE blend was attributable to the additive effects of genes.

Heritability of responsiveness measured as the sum of the responses to the 44% ZE blend and to an off-blend (either 25 or 65% ZE) was 0.326 ± 0.085 (significantly different from zero, $t = 3.83$, $N = 173$). The estimated heritability for the combined response is similar to the estimate obtained using the natural blend alone. However, when males exposed to the 25 and 65% ZE blends are considered separately, a pronounced asymmetry is apparent. The heritability of combined responses to the 44 and 25% ZE blends was 0.542 ± 0.103 ($t = 5.273$, $N = 173$; $P < 0.01$). The heritability of response duration to the 25% ZE blend alone was also significant ($h^2 = 0.377 \pm 0.113$, $t = 3.341$, $N = 94$; $P < 0.01$) (Figure 2a). However, the comparable estimate for the summed responses of males exposed to 44 and 65% ZE blends was 0.064 ± 0.137 ($t = 0.465$, $N = 173$; $P > 0.05$). This is a result of the nonsignificant heritability of response to the 65% ZE blend ($h^2 = -0.145 \pm 0.103$, $t = 1.404$, $N = 79$; $P > 0.05$) (Figure 2c).

Heritability of Response Specificity. Heritability of response specificity was 0.146 ± 0.045 ($t = 3.273$, $N = 177$; $P < 0.01$). However, due to the asymmetry in response to the 25 and 65% ZE blends, the heritability of response specificity for males exposed to these blends differed substantially. The heritability for males exposed to the 25% ZE blend was 0.117 ± 0.059 ($t = 1.99$, $N = 98$; $P < 0.05$), while the heritability for males exposed to the 65% ZE blend was -0.043 ± 0.067 ($t < 1$, $N = 78$; $P > 0.05$).

Latency. The estimated heritability of the latency associated with wing fanning to the 44% ZE blend was 0.027 ± 0.109 and was not significantly different from zero ($t = 0.245$, $N = 166$; $P > 0.05$). The low heritability of latency is partly due to a high degree of variability associated with this response measure. The mean (\pm SD) latencies of males responding to the 44% ZE blend in the parental and F_1 generations were 7.97 ± 13.34 and 10.33 ± 16.63 sec, respectively. The coefficients of variation (CV) for these estimates are 167.38

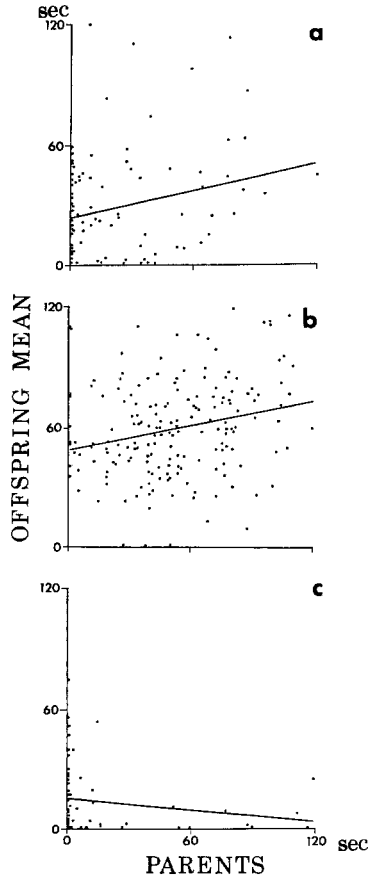


FIG. 2. Regression of wing-fanning duration to pheromone blends containing (a) 25%, (b) 44%, or (c) 65% (*Z, E*)-7,11-hexadecadienyl acetate by male parents on the mean wing-fanning durations of their sons.

and 160.99. Corresponding CV estimates for the duration of response to the 44% *ZE* blend were 51.4 and 49.3% for the parental and F_1 generations, respectively.

Threshold. Pheromone response threshold was measured by the latency of wing fanning by males exposed to increasing pheromone concentration series. Mean latencies (\pm SD) for the 25 and 65% *ZE* blends were 79.38 ± 53.24 and 102.98 ± 62.37 sec, respectively, for males in the parental generation. Comparable means for the offspring of these males were 96.14 ± 41.03 and 113.58 ± 45.27 for the low and high *ZE* blends, respectively. The latencies in the

parental and F_1 generations were not significantly different for the stimulus series involving the 25% ZE blend (Mann-Whitney U test; $t_s = 1.56$; $P > 0.05$) but were different for the 65% ZE series (Mann-Whitney U test; $t_s = 2.11$; $P < 0.05$).

Heritabilities (\pm SE) of threshold were 0.113 ± 0.175 and 0.217 ± 0.164 for the 25 and 65% ZE stimuli, respectively, and were not significant ($t = 0.644$ and $t = 1.325$, $N = 58$, $P > 0.05$). Heritability of the summed latencies for both stimuli was 0.110 ± 0.131 (not significant, $t = 0.844$, $N = 58$; $P > 0.05$).

Heritability of threshold asymmetry (measured by the difference between latencies for the two stimuli series) was 0.314 ± 0.23 (not significant, $t = 1.33$, $N = 58$, $P > 0.05$). Thus, variance in threshold (measured by response latency) upon which either natural or artificial selection may act was not found in our laboratory colony.

DISCUSSION

Selection may reduce additive genetic variance in characters closely related to fitness, because alleles that increase fitness will become fixed as a population approaches equilibrium (Fisher, 1958). Thus, heritabilities for characters closely associated with reproductive success are typically low (Falconer, 1981). For example, Gustaffsson (1986) found an inverse relationship between heritability and the influence on fitness for a number of morphological and life history characters in the collared flycatcher (*Ficedula albicollis*). Particular components of fitness may be heritable, but negative genetic correlations between them tend to reduce the heritability of lifetime reproductive success (Jones, 1987), although positive correlations have also been documented (Palmer and Dingle, 1986).

Male mating behavior is generally considered to be an important component of fitness (Ehrman and Parsons, 1981). Nonetheless, significant heritable variation in both overall responsiveness and in response specificity has been demonstrated for a laboratory population of the pink bollworm. A number of mechanisms have been suggested to account for the persistence of additive genetic variance, including mutation (Lande, 1976), linkage disequilibrium (Taylor and Williams, 1982), variable environments (Cade, 1984), genetic drift, and immigration (Jones, 1987). However, heritability estimates must be interpreted carefully because they are strictly applicable only for the population used in making the estimates and for the particular set of behavioral and ecological circumstances in which the measurements were made. Further, heritability estimates assume negligible genetic-environmental interactions, dominance and epistatic effects, genetic drift, and natural selection.

Evolutionary change in male response could occur through an altered pro-

pensity to respond to all blends or through a change in the pattern of relative responses to particular blends. The heritability of overall responsiveness by males observed in this study (0.385) is similar to the estimated heritability for the amount of total pheromone produced by females (0.410) (Collins and Cardé, 1985). These estimates demonstrate that there is heritable variance associated with quantitative aspects of both signal production and response. It has been suggested that females emitting little pheromone may tend to attract males that have low response thresholds (Greenfield, 1981). On the other hand, resistance to synthetic pheromone used as a mating disruptant could evolve by increased emission rates (Cardé, 1976, 1981; Haynes et al., 1984). Male response to inappropriate stimuli (such as a low background concentration of formulated pheromone) could be minimized by an elevated response threshold, although the same end might be achieved by behavioral habituation *sensu lato*.

A shift in the ratio of pheromone components emitted also has been proposed as a qualitative mechanism for the evolution of resistance to synthetic pheromone (Cardé, 1976, 1981). The ratio produced by pink bollworm females has a significant heritability (0.342) (Collins and Cardé, 1985). However, interpretation of the degree of genetically based variation in male response specificity is complicated by an asymmetry in the heritabilities of responses to ratios on either side of the natural mean.

Males were more responsive to the 25% *ZE* blend than to the 65% blend, and heritable variation was found for the 25% *ZE* blend, but not for the 65% *ZE* blend. A similar asymmetry in the heritability of response specificity was observed. Thus, the ratio evoking maximum response could be shifted in males toward a blend with less *ZE* isomer, but selection in the other direction should be more difficult. Genetic influence on variation may be greater for dose–blend combinations that elicit a greater response, but the relationship between response level and heritability is not known.

Heritability estimates in this study were derived using blends of a single concentration at a constant temperature. However, blend preference patterns may shift with concentration (Linn and Roelofs, 1985), and response specificity can be modulated by temperature (Linn et al., 1988). Therefore, a full understanding of the response asymmetry would require heritability estimates for a range of pheromone doses at several temperatures.

Although the pink bollworm was originally described from specimens collected in India, it is believed to be indigenous to Australia (Flint et al., 1979). Attraction to different blends of (*Z*, *E*)- and (*Z*, *Z*)-7,11-hexadecadienyl acetate has been examined for three closely related *Pectinophora* species in Australia (Rothschild, 1975). Based on pheromone trap-catch data, *P. gossypiella*, *P. endema* Common, and *P. scutigera* (Holdaway) were maximally responsive to blends with 50, 33, and 10% *ZE*, respectively. Where these species are sym-

patric, a reduction in heritable variance in the responsiveness of pink bollworms to blends with a lower percentage of *ZE* might be expected.

Based on the relatively high degree of heritable variation in overall male responsiveness reported here, and the estimated heritability previously observed for pheromone titer in females (Collins and Cardé, 1985), rapid changes in quantitative features of the pheromone communication system of the pink bollworm appear feasible under appropriate selection pressures, such as the use of formulated synthetic pheromone to disrupt mating.

APPENDIX A

Response Specificity. The specificity of wing-fanning to different pheromone blends was measured by first computing the relative responses to the normal (44% *ZE*) and off-blends (25 or 65% *ZE*) for each male. Relative response to the normal blend, R_n , was defined as:

$$R_n = D_n / (D_n + D_o) \quad (1)$$

where D_n and D_o are the durations of wing-fanning to the normal and off-blend, respectively. Relative response to the off-blend, R_o , was thus:

$$R_o = 1 - R_n \quad (2)$$

Specificity (S) was then estimated by comparing relative responses using a modification of the Shannon-Weaver index (Shannon, 1949):

$$S = \begin{cases} [-(R_n \log_e R_n + R_o \log_e R_o)] / 1.386, & R_n \leq 0.5 \\ [1.386 + (R_n \log_e R_n + R_o \log_e R_o)] / 1.386, & R_n > 0.5 \end{cases} \quad (3)$$

Equation 3 is partitioned into two ranges to permit differentiation between males that respond predominantly to the normal blend and males that respond primarily to an off blend. Thus, if the duration of wing-fanning is longer for the normal than for the off-blend, S will be greater than 0.5, whereas S for males that are more responsive to the off-blend will be less than 0.5 (Figure 1). For example, if the duration of wing fanning to 25 and 44% *ZE* blends were 10 and 30 sec, respectively, then R_n would equal 0.75 [=30/(10 + 30)], $R_o = 0.25$ (=1 - 0.75), and $S = 0.594$ {=[1.386 + (0.75 log_e 0.75 + 0.25 log_e 0.25)/1.386]}. If the responses were reversed (30 and 10 sec for the 25 and 44% *ZE* blends), then R_n would equal 0.25, $R_o = 0.75$, and $S = 0.406$ {=[-(0.25 log_e 0.25 + 0.75 log_e 0.75)/1.386]}. Because specificity is undefined for males that did not respond to at least one of the stimuli, families were excluded from the analysis if the male parent or all male offspring failed to respond to either blend.

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USE OF ION-PAIRING REVERSED-PHASE LIQUID CHROMATOGRAPHY IN SEPARATION OF SOLANIDINE AND SOLASODINE

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Abstract—A method based on ion-pairing reversed-phase liquid chromatography was developed to separate and quantify the total alkaloid content in *S. ptycanthum* (eastern black nightshade). The method is shown to separate satisfactorily the aglycones and some of the glycoalkaloids. The mobile phase used was aqueous sodium dodecyl sulfate-acetonitrile.

Key Words—*Solanum ptycanthum*, eastern black nightshade, glycoalkaloids, ion-pairing chromatography, solanidine, solasodine.

INTRODUCTION

Solanum ptycanthum (known as eastern black nightshade) is a problem weed in North America. Many crop fields, such as soybean, wheat, and sunflower, are infested with this weed. Black nightshade has a high content of steroidal alkaloids. Since alkaloids are known to be germination inhibitors (Evenari, 1949), it is of interest to study and evaluate them as potential means for weed control.

Early work relating to the identification, separation, and quantification of alkaloids was done using thin-layer chromatography (TLC) (Paquin and Lepage, 1963). The TLC method, however, was not always successful in separating the alkaloids. More recently, high-performance liquid chromatography (HPLC) has been used. Bushway et al. (1979) succeeded in separating the glycosides α -chaconine, β -chaconine, and α -solanine by using three different columns. Crabbe and Fryer (1980) analyzed and separated solasodine, solanine, solamargine, and solasodiene using reversed-phase partition chromatography. Hunter et al. (1980) used adsorption chromatography on a silica column for the

separation of *Solanum* and *Veratrum* alkaloids. Hunter and Heftmann (1983) fractionated *Solanum laciniatum* leaves using the chromatotron and analyzed the glycoalkaloid obtained by reversed-phase HPLC. Cham and Wilson (1987) used mass spectral analyses to analyze the glycoalkaloids extracted from *Solanum sodomaeum*. Eldridge and Hockridge (1983) identified the glycoalkaloids present in eastern black nightshade dried berries (*Solanum ptycanthum*). These studies showed that HPLC is a more reliable and easier method than TLC to identify and quantify the alkaloid content in various plant extracts. Most of the investigations, however, dealt with the glycoalkaloids.

In this study, a simpler and more rapid method for quantifying the total alkaloid content was developed. The method initially hydrolyzes the *Solanum ptycanthum* glycoalkaloids to their aglycones and then uses ion-pairing reversed-phase chromatography to separate and quantify the total alkaloid content. The advantages of this method are: (1) the aglycone is more stable, (2) the aglycone is more easily identifiable, and (3) the standards for aglycones are more readily available.

METHODS AND MATERIALS

Fresh green berries from *Solanum ptycanthum* picked locally were ground and extracted with boiling methanol, filtered, and concentrated to the desired volume. A portion of the sample was taken and diluted, if necessary, with methanol. For the detection of glycoalkaloids, this portion was injected in the HPLC along with the standards. Another portion of the original extract was hydrolyzed, according to Eldridge and Hockridge (1983), and the aglycone was extracted using chloroform. Then 10 μl of the chloroform extract was injected into the HPLC.

The HPLC used has an Isco V_4 absorbance detector, a Milton Roy mini-pump having a pulse dampener, a Valco injection valve, and Alltech C_{18} column (10 μm particle size, 25 cm long, and 4.6 mm ID). Tomatidine, solanidine, α -solanine, α -chaconine, and solasodine standards were purchased from Sigma Chemical Company, (St. Louis, Missouri). Solamargine was generously supplied by Dr. S.L. Sinden, U.S. Department of Agriculture, Beltsville, Maryland. All standards were dissolved in methanol except for solanidine, which was dissolved in chloroform. The concentration of all the standards was 0.2–0.3 mg/ml. All solvents and reagents were analytical grade or HPLC grade. The wavelength used was 205 nm, sensitivity 0.02 a.u.f.s, and the flow rate of the solvent was 1.4 ml/min. The solvent used for the ion-pairing reversed-phase chromatography method was 0.017 M (0.5% w/v) aqueous sodium dodecyl sulfate–acetonitrile (15:85). Going to a 1% concentration did not improve the resolution. The aqueous sodium dodecyl sulfate was adjusted to pH 2.3–2.5

with 85% phosphoric acid before mixing it with the acetonitrile. Severe peak broadening was observed at pH higher than 3. Sodium dodecyl sulfate was chosen because it is a commonly available counter ion and is transparent at the wavelengths used. Acetonitrile was used because it usually gives better column efficiency than methanol and is also transparent at short wavelengths. The C₁₈ column was used because it gave better separation of the alkaloids than the C₈ column.

RESULTS AND DISCUSSION

It is difficult to find standards for some glycoalkaloids. Furthermore, the use of crude extract in the separation and detection of alkaloids is easier and faster than if purified extract is required. It would be useful, therefore, to find a method that resolves these two difficulties. Ion-pairing reversed-phase chromatography is such a method. It uses hydrolyzed crude extract, which is more stable, and relies on the aglycone whose standard is more readily available. Moreover, the determination of the alkaloid aglycones avoids the coextractants commonly interfering with the glycoside separation and the variation in glycoside patterns resulting from decomposition of extracted material.

Figure 1 shows the combined standard for solanidine and solasodine co-chromatographed. The figure shows the solanidine standard to be eluted in about 18 min and the solasodine standard by about 24 min. Figure 1 shows that the two standards are clearly separated in approximately 5 min. Moreover, the two peaks are well defined with no interference.

Figure 2 is a chromatogram of the hydrolyzed crude extract of *Solanum ptycanthum* fresh green berries. This chromatogram was obtained using the same conditions as those for the standards in Figure 1. Figure 2 clearly shows the presence of the aglycone solasodine in the *Solanum ptycanthum* green berries extract. The sample does not appear to contain any solanidine.

Chromatograms were also run on tomatidine, the aglycone of tomatine, found mainly in tomatoes, using ion-pairing reversed-phase chromatography. It was found that a much larger quantity of the standard, 5 μ g or more should be injected into the HPLC to obtain an appreciable peak. The retention time was found to be intermediate between solanidine and solasodine—about 22.7 min.

To check whether the ion-pairing reversed-phase chromatography method also works for glycoalkaloid separation, some available glycoalkaloid standards were tested in the same way. Solamargine and α -solanine were cochromatographed. Figure 3 shows that they were successfully separated. The resolution of α -chaconine and α -solanine was poor when the two standards were cochromatographed. The same poor result was obtained with solamargine and α -chaconine.

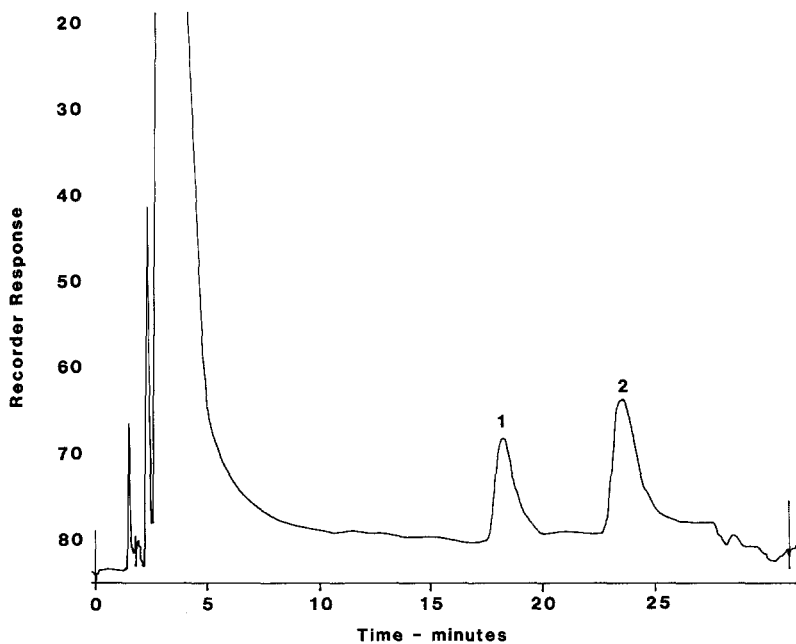


FIG. 1. Chromatogram of mixture of solanidine and solasodine standards. Flow rate 1.4 ml/min; detector sensitivity, 0.02 a.u.f.s.; wavelength, 205 nm; chart speed, 6.6 mm/min; 2 μ g injection. Peak 1 = solanidine; peak 2 = solasodine.

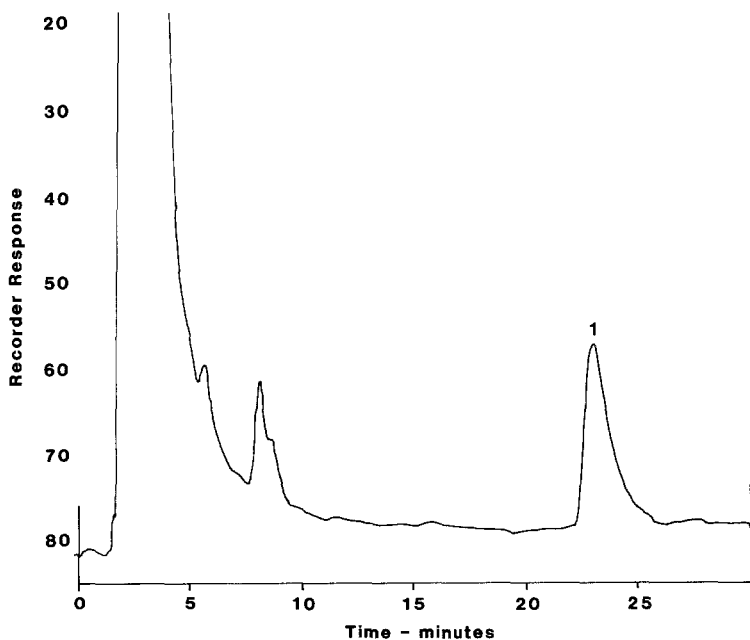


FIG. 2. Chromatogram of hydrolyzed *Solanum ptycanthum* berries. Flow rate 1.4 ml/min; detector sensitivity, 0.02 a.u.f.s.; wavelength, 205 nm; chart speed, 6.6 mm/min; 10 μ l injection. Peak 1 = solasodine.

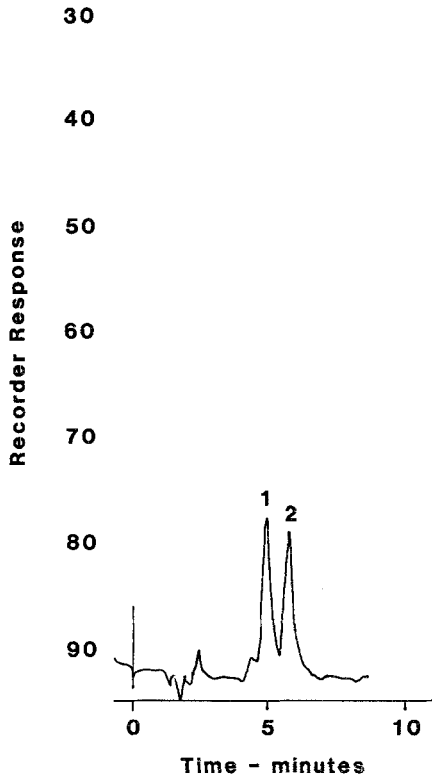


FIG. 3. Chromatogram of mixture of solamargine and α -solanine. Flow rate 1.4 ml/min; detector sensitivity, 0.02 a.u.f.s.; wavelength, 205 nm; chart speed, 6.6 mm/min; 10 μ l injection. Peak 1 = solamargine; peak 2 = α -solanine.

CONCLUSIONS

The aglycones solanidine and solasodine can be separated and identified by ion-pairing reversed-phase chromatography. Tomatidine, solasodine, and solanidine are resolved when all three are present in the same mixture. Some of the glycoalkaloids were separated. This method could also be used to separate aglycones from glycoalkaloids when they are present in the same mixture. It is suggested that the column used in ion-pairing experiments be used only for that purpose. Finally, it is easier and faster to quantify total alkaloid content as aglycones rather than as glycoalkaloids.

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ACTIVATION OF PLANT FOLIAR OXIDASES BY INSECT FEEDING REDUCES NUTRITIVE QUALITY OF FOLIAGE FOR NOCTUID HERBIVORES

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Abstract—The foliage and fruit of the tomato plant *Lycopersicon esculentum* contains polyphenol oxidases (PPO) and peroxidases (POD) that are compartmentally separated from orthodihydroxyphenolic substrates in situ. However, when leaf tissue is damaged by insect feeding, the enzyme and phenolic substrates come in contact, resulting in the rapid oxidation of phenolics to orthoquinones. When the tomato fruitworm *Heliothis zea* or the beet armyworm *Spodoptera exigua* feed on tomato foliage, a substantial amount of the ingested chlorogenic acid is oxidized to chlorogenoquinone by PPO in the insect gut. Additionally, the digestive enzymes of the fruitworm have the potential to further activate foliar oxidase activity in the gut. Chlorogenoquinone is a highly reactive electrophilic molecule that readily binds covalently to nucleophilic groups of amino acids and proteins. In particular, the —SH and —NH₂ groups of amino acids are susceptible to binding or alkylation. In experiments with tomato foliage, the relative growth rate of the fruitworm was negatively correlated with PPO activity. As the tomato plant matures, foliar PPO activity may increase nearly 10-fold while the growth rate of the fruitworm is severely depressed. In tomato fruit, the levels of PPO are highest in small immature fruit but are essentially negligible in mature fruit. The growth rate of larvae on fruit was also negatively correlated with PPO activity, with the fastest larval growth rate occurring when larvae fed on mature fruit. The reduction in larval growth is proposed to result from the alkylation of amino acids/protein by *o*-quinones, and the subsequent reduction in the nutritive quality of foliage. This alkylation reduces the digestibility of dietary protein and the bioavailability of amino acids. We believe that this mechanism of digestibility reduction may be extrapolatable to other plant-insect systems because of the ubiquitous cooccurrence of PPO and phenolic substrates among vascular plant species.

Key Words—Polyphenol oxidase, peroxidase, digestibility reduction, plant-insect interactions, phenolic-protein binding, chlorogenic acid, *Heliothis zea*, *Spodoptera exigua*, *Lepidoptera*, *Noctuidae*, *Lycopersicon esculentum*, host-plant resistance.

INTRODUCTION

Phenolic compounds, ranging from simple C₆-C₁ and C₆-C₃ forms to more complex polymers such as tannins and lignins, are widely distributed among vascular plants (Harborne, 1982). Many phenolics are thought to function as chemical defenses against herbivory, in part because of their ability to interact with proteins and inhibit enzyme functions (Singleton, 1981; Feeny, 1976; Rhoades, 1983; Coley et al., 1985). Current ecological theories ascribe the digestibility-reducing properties of quantitative defenses to many phenolic compounds such as lignans and tannins (Coley et al., 1985; Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1985). Abundant literature citations exist concerning the ability of phenolics to function as both inducible and constitutive defenses against herbivory (Schultz and Baldwin, 1982; Rhoades, 1983; Coley et al., 1985; Zucker, 1982; Lincoln, 1985; Lindroth and Peterson, 1988). However, a paucity of data exists that demonstrate phenolics such as tannins function as digestibility-reducing defenses against insect herbivores (J.S. Martin et al., 1987).

Much of the evidence for the defensive role of phenolics is derived from *ex planta* bioassays; that is, the candidate phenolic compound is isolated from foliage and incorporated into the artificial diet of the insect and its effects on herbivore feeding behavior, growth and/or survival are determined. This mechanistic approach has been applied to various agricultural crops such as cotton (e.g., Hedin et al., 1983; Chan et al., 1978), tomato (Isman and Duffey, 1982a,b; Elliger et al., 1981), and corn (Waiss et al., 1979). Alternatively, some investigators have relied upon a more holistic approach wherein the concentration of a given phenolic may be correlatable with insect populations and/or resistance under more natural, field conditions (Lincoln, 1985; Zucker, 1982; Widstrom et al., 1982). These approaches are invaluable in improving our understanding of the role of plant phenolics in insect-plant interactions. However, both approaches may be inadequate in determining cause-effect in situations where the effect of a given phytochemical is dependent upon other factors such as:

1. The dynamic state of the phenolic both within the plant and within the herbivore. For example, some authors have shown that insect feeding induces changes in the phenolic metabolism of the host plant, which may then alter the defensive quality of the plant (Chiang et al., 1987; Schultz and Baldwin, 1982;

Rhoades, 1983; Loper, 1968; Thielges, 1968; Haukioja and Niemela, 1977). Certain phenolic glycosides such as glucoside of DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one) are hydrolyzed by glucosidases following tissue damage by herbivores to release the aglycone, which is the primary active agent against insects (Klun et al., 1967).

2. The greater chemical context of the host-plant. For instance, when phenylalanine was nutritionally limiting to certain herbivores, dihydroxyphenols appeared not to function as plant defenses but instead functioned as nutrients by sparing phenylalanine in the production of cuticular tanning agents (Bernays et al., 1982). The toxicity of gossypol to the boll weevil was ameliorated by increased dietary protein (Moore, 1983). Conversely, Duffey and Bloem (1986) reported that the toxicity of the phenolic, rutin, was enhanced by increasing the concentration of dietary protein.

3. The physiological and/or chemical properties of the gut environment of the specific herbivore. The ultimate fate of tannins in the gut environment of the herbivore is crucial to understanding the potential defensive nature of these phenolics (Bernays, 1981; M.M. Martin and Martin, 1984; J.S. Martin et al., 1987). Binding of tannins to protein is determined by a number of plant- and herbivore-related factors such as the pH of the insect digestive fluid, the presence of surfactants in the digestive system, and the specific structures of both the protein and the tannin (Berenbaum, 1980; J.S. Martin et al., 1987; Zucker, 1983; Hagerman and Butler, 1981; Asquith and Butler, 1986). Because these factors may inhibit tannin-protein complexes, several authors have argued against digestibility reduction as a mode of action for tannins in insect herbivores and have, instead, claimed that tannins act as feeding deterrents or toxins (Bernays, 1981; J.S. Martin et al., 1987; Berenbaum, 1983).

The interaction of phenolics with protein may be a critical factor in understanding the ability of phenolics to act as plant defenses. Other plant phenolics, in addition to tannins, are known to interact chemically with protein. Phenolics may react with protein in four different ways: the formation of tannin-protein complexes via hydrogen bond interactions, valence forces such as hydrophobic interactions, formation of Schiff bases (e.g., gossypol), and alkylative reactions via the oxidation of orthodihydroxyphenolics to orthoquinones by polyphenol oxidases or peroxidases to form covalent bonds with the nucleophilic groups of amino acids (Pierpoint, 1983; Matheis and Whitaker, 1984; McManus et al., 1983; Singleton, 1981). Because phenolic compounds are known to interact chemically with proteins and to serve as substrates for a number of plant hydrolytic and oxidative enzymes (Butt, 1981; Butt and Lamb, 1981), it is imperative that the toxicity of these compounds be assessed under conditions more representative of their *in situ* occurrence within the host plant. Reliance solely on artificial diet experiments or solely on correlational field studies may yield mis-

leading conclusions regarding both the mode of action and potential function of these plant compounds as bases of host-plant defense.

The tomato plant *Lycopersicon esculentum* contains several *o*-dihydroxyphenols, including chlorogenic acid, rutin, and caffeoyl-aldaric acid, which have been shown to inhibit the growth of noctuid larvae when incorporated into artificial diets (Duffey and Isman, 1981; Isman and Duffey, 1982a; Elliger et al., 1981). However, when *Heliothis zea* larvae were grown on excised tomato leaflets, their growth was not correlatable with the foliar concentration of *o*-dihydroxyphenols (Isman and Duffey, 1982b). Implicit in this finding was that the toxicity of these phenols may be dependent upon the chemical context of the host plant, that is, the interaction of the phenolic with other phytochemicals in the foliage.

A component of foliage that has been unaccounted for in the tomato–noctuid system and may be crucial to understanding the toxicity of *o*-dihydroxyphenolics is the presence of foliar oxidative enzymes. The tomato plant contains polyphenol oxidases (PPO) and peroxidases (POD), which are compartmentalized apart from their phenolic substrates *in situ* but have the ability to rapidly oxidize *o*-dihydroxyphenolics to the corresponding *o*-quinone when leaf tissue is damaged (Duffey, 1986). In particular, chlorogenic acid is a substrate for these enzymes and is rapidly converted to chlorogenoquinone, a highly reactive molecule that is known to covalently bind to nucleophilic $-\text{NH}_2$ and $-\text{SH}$ groups of molecules such as amino acids and proteins (Pierpoint, 1966, 1969, 1983; Hurrell et al., 1982; Matheis and Whitaker, 1984). This alkylative binding of chlorogenoquinone to amino acids may reduce leaf protein digestibility *in vitro* (Free and Satterlee, 1975; Barbeau and Kinsella, 1985; Horigome and Kandatsu, 1968) and/or the bioavailability of essential amino acids to the herbivore (Hurrell et al., 1982; Igarashi and Yasui, 1985). If a significant amount of the ingested chlorogenic acid is oxidized when insect larvae feed on foliage containing polyphenol oxidases (PPO) or peroxidases (POD), the effect of the phenolic on the insect may be profoundly different from its effect in a reduced state. In an oxidized state, the ultimate effect and fate of chlorogenic acid in the herbivore depends upon foliar oxidative activity and the relative amounts of the reactants including chlorogenic acid and available $-\text{SH}$ and $-\text{NH}_2$ groups. For this reason, a better understanding of phenolic toxicity will emerge if assessed under conditions approximating those that occur *in situ*.

In this paper, we determined if chlorogenic acid is oxidized when the generalist larval noctuids *Heliothis zea* and *Spodoptera exigua* ingest tomato foliage, and what, if any, consequence this oxidation may have on the growth of these insects. Additionally, we examined the relationship of phenological changes in the concentrations of foliar oxidases and phenolic concentrations with the ability of foliage to support larval growth.

METHODS AND MATERIALS

Quantification of Phytochemicals. The concentrations of *o*-diphenols, rutin and chlorogenic acid, were determined colorimetrically at OD₃₉₀ and OD₄₄₀ using 0.5% diphenylborate-ethanolamine complex (Broadway et al., 1986). In the experiments with leaflet bioassays, phenolic concentrations were determined using two leaflets per leaf. The variation in the phenolic concentration between the two leaflets used for phenolic determination and the three leaflets used for the bioassay averaged less than 10% when leaflets from the same leaf were used ($N = 20$).

The concentration of leaf protein was determined using the Coomassie brilliant blue reagent (Bio-Rad Laboratories, Richmond, California) after Bradford (1976) and Snyder and Desborough (1978) with bovine serum albumin as a standard. The concentration of free amino groups in the foliage was determined colorimetrically with TNBS (2,4,6-trinitrobenzenesulfonic acid) following the method of Fields (1972). An 80% ethanolic extract of the foliage was partitioned against an equal volume of chloroform. The ethanolic fraction containing free amino acids was evaporated and reconstituted with 0.1 M NaBO₄ and analyzed with TNBS.

Measurement of Polyphenol Oxidase and Peroxidase Activity. To isolate enzyme from foliage, individually weighed leaflets (ca. 300 mg wet weight) were homogenized in 2.5 ml of 0.1 M potassium phosphate buffer, pH 6.8, containing 7% (w/v) polyvinylpyrrolidone (PVPP). Then 0.8 ml of 10% (v/v) Triton X-100 was added and the sample was vortexed for 10 sec. The sample was centrifuged at 11,000g for 10 min, and the supernatant was used immediately as the enzyme source. Polyphenol oxidase activity (as CHA oxidase) was measured spectrophotometrically (Ryan et al., 1982) by recording the increase in OD₄₇₀. Activity was expressed as the change in OD₄₇₀ $\times 10^{-2}$ /min/g wet weight unless otherwise indicated. Peroxidase activity was measured identically except with the addition of 0.1 ml 3% H₂O₂. Also, both PPO and POD activity were initially determined with chlorogenic acid, caffeic acid, hydroquinone, *p*-coumaric acid, *p*-hydroxybenzoic acid, tyrosine, and guaiacol as potential substrates in the presence and absence of exogenous hydrogen peroxide (Ryan et al., 1982).

The effect of different pHs on enzymatic activity was determined by homogenizing leaf material in 0.1 M sodium acetate or potassium phosphate buffer at pH ranging from 4.0 to 10.0. Subsequent treatment of samples was as described and PPO activity was determined at the respective pH.

The PPO/POD activity (without added H₂O₂) of fruit from field-grown tomato plants described below was determined. Enzyme from fruit was isolated as from leaflets except that a single plug of mesocarp and endocarp tissue (ca.

100 mg fresh weight) was removed from each fruit. Fruit ranged from immature green (ca. 2 g fresh weight) to mature red (> 100 g fresh weight).

Insects. Larval *S. exigua* and *H. zea* were reared on artificial diet (Chippendale, 1970) at 25°C with a photoperiod of 16:8 hr light-dark after Broadway and Duffey (1986a).

Bioassays with Foliage and Fruit. Foliage from greenhouse-grown or field-grown *L. esculentum* var. Castlemart was used in the bioassays. To determine if oxidation of phenolics in tomato foliage reduces the growth rate of *S. exigua*, leaflets were excised from greenhouse plants at the six- to eight-leaf stage and transported to the laboratory on ice.

Treatment I was the oxidized treatment where PPO inhibitors were not added to the foliage until substantial oxidation had occurred. For this treatment, 100 g fresh wt foliage was macerated in a blender for 2 min in 200 ml of ice-cold ddH₂O, transferred to a beaker, and mechanically stirred for 2 hr at room temperature. The 2-hr stirring period was chosen because it represents the minimal time that larvae retain tomato foliage in their digestive system (unpublished data), although >90% of tomato PPO activity occurs during the first 15 min following maceration. After stirring for 2 hr to allow oxidation, the PPO inhibitor phenylthiourea (50 mg) and reducing agent ascorbic acid (150 mg) were added, and the solution was stirred for an additional 10 min. The solution containing the foliage and PPO inhibitors was frozen and lyophilized. Dried foliage was reconstituted in H₂O and agar to the original weight of 100g and fed to *S. exigua* larvae.

Treatment II, the treatment without oxidation, was similar but with the following exceptions: The foliage was macerated for 2 min in a blender with ice-cold ddH₂O containing 50 mg of phenylthiourea and 150 mg of ascorbic acid. The solution was transferred to a beaker and stirred for 2 hr and 10 min. A diet was prepared from foliage as described above. The concentrations of protein, free amines, *o*-dihydroxyphenolics, and PPO activity in samples of leaf material were measured before treatment (maceration) and after treatment as described. Ten neonate *S. exigua* larvae were placed in each paper cup containing the appropriate diet with five cups (replicates) per treatment. Diet was supplied ad libitum, larvae were weighed daily to the nearest 0.1 mg for 13 days, and relative growth rates were determined following Waldbauer (1968). Differences between treatments were determined by Student's *t* test.

To determine the effect of plant age on the ability of foliage to support the growth of noctuid larvae, leaflets from greenhouse- and field-grown tomato plants were utilized. For the experiments with greenhouse plants, 20 tomato plants (var. Burpee VFN) were grown in 1-gallon containers. Leaflets were excised from five to six plants at three different growth stages: preflowering, flowering, and green fruit stage. Three weighed leaflets from each leaf were placed in a Petri dish containing a single third-instar *H. zea* (one day postec-

dysis). After 72 hr, insect and leaf weight were recorded and the relative growth rate (RGR) was computed after Waldbauer (1968). The remaining 4 leaflets from each leaf were used for quantification of PPO and phenolics according to methods described. Leaflets used for bioassay or phytochemical analysis were removed from the same position on a leaf throughout the experiment. Fifteen larvae were used per plant stage.

To determine the impact of seasonal variation of field-grown plants on larval growth rates, 400 tomato plants were grown in the field during the summer of 1987 (University of California at Davis research plots). Seeds of *L. esculentum* var. Castlemart were planted in 100-ml plastic pots in the greenhouse. When plants reached the 1–2 leaf stage, they were transplanted to the field and irrigated weekly. Two fully expanded terminal leaves per plant were excised for bioassays from plants at the flowering, immature green fruit, mature green fruit, and ripe fruit stages. Three leaflets per leaf were fed to larvae as described above and RGRs were computed following Waldbauer (1968). Quantification of PPO activity was made from one of the remaining four leaflets from each leaf as described above. Leaflets for PPO analysis or bioassay were removed from the same position on the leaf throughout the assays. A minimum of 50 larvae per plant stage was tested.

Bioassays were also conducted with tomato fruit from various stages of development, including ripe (> 100 g); mature green (> 100 g); large immature green (50–100 g); medium immature green (10–49.9 g), and small immature green (2–9.9 g). Samples from each of these categories were analyzed for PPO activity as described above ($N = 5$ /stage). Bioassays were conducted with third instar *H. zea* in 500-ml beakers covered with gauze. The RGRs of larvae were determined as previously described and a minimum of six larvae per fruit stage were tested.

Bioassays with Artificial Diet. To determine if the effect of oxidation of chlorogenic acid resulted from the formation of a polymer, the alkylation of dietary protein, or the reduction in availability of free amino acids, experiments with artificial diets (Chippendale, 1970) were conducted. In the first treatment, where oxidized chlorogenic acid was not allowed to interact with dietary protein so that only a polymer was formed, CHA at 3.5 $\mu\text{mol/g}$ diet and PPO (= tyrosinase, Sigma Chemical Co., St. Louis, Missouri) at 0.100 $\text{OD}_{470}/\text{min/g}$ diet of CHA oxidase activity were incubated for 2 hr in ddH_2O and added to the remaining diet ingredients, which contained 0.5% (w/w) casein. In the second treatment, CHA, PPO, and casein were incubated simultaneously so that the casein was alkylated by the quinone. In the third treatment, an enzymatic digest of casein containing only TCA-soluble amines and/or peptides (Sigma Chemical Co) was incubated at 0.5% (w/w) with CHA and PPO for 2 hr, and then added to diet. This treatment involved alkylation of small peptides and free amino acids instead of protein. Treatment diets were fed to neonate *S. exigua*

ad libitum for 12 days and consisted of at least three replicates of 10–15 larvae per treatment with total of $N = 336$ for the experiment. Larval weights were recorded to the nearest 0.1 mg on the 12th day and compared to the appropriate control containing no added phytochemicals (Broadway and Duffey, 1986b).

Amino Acid Analyses. To determine the effect of alkylation on the amino acid composition of leaf protein, protein was first isolated from foliage by macerating for 2 min in 0.1 M potassium phosphate buffer, pH 7.0, containing 10^{-4} M phenylthiourea (PTU) to inhibit PPO activity. The homogenate was filtered through cheesecloth and Whatman No. 1 filter paper, followed by acid precipitation at pH 3.5, and centrifugation at 10,000g for 15 min. The protein pellet was dialyzed for 48 hr against ddH₂O, frozen, and lyophilized.

The lyophilized protein was given one of four treatments. In treatment 1, 1.2 g of protein was incubated with 320 mg of chlorogenic acid and 3.2 mg of PPO (mushroom tyrosinase, Sigma Chemical Co.) in 100 ml 0.1 M potassium phosphate buffer, pH 7.0. After 2 hr, the reaction was stopped with 5 mg of PTU. In treatment 2, the PTU was preincubated with the tyrosinase to inactivate the enzyme prior to incubation with CHA and protein. In treatment 3, the effect of polyphenol oxidase from tomato foliage on tomato protein was determined as follows. An enzyme solution was obtained (described above) and incubated with 1.2 g tomato protein and 320 mg chlorogenic acid as described. The enzyme solution, in addition to containing polyphenol oxidase, was contaminated with significant amounts of free amino acids (ca. 0.7% w/w following Fields, 1972). In treatment 4, the PTU was preincubated with the tomato PPO prior to incubation with protein. Therefore, the effect of oxidation of CHA on proteinaceous and free amino acids was determined in treatments 3 and 4. Immediately following incubation, each treatment was then frozen and lyophilized. The amino acid composition of the four treated proteins was determined using a Beckman amino acid analyzer after protein had been hydrolyzed with 6 N HCl for 24 hr in sealed tubes in vacuo. Kjeldahl nitrogen was determined using a micro-Kjeldahl method.

Determination of Polyphenol Oxidase Activity in Feces and Regurgitate of Larvae. Third-instar *H. zea* larvae were placed on artificial diet containing 3.5 μ mol CHA/g or on tomato foliage (Castlemart) for 24 hr. Freshly produced feces from 100–125 larvae per treatment were collected (ca. 1.0 g total weight) during the last 4 hr of the experiment and were placed immediately on ice and homogenized with 1 ml 0.1 M potassium phosphate, pH 8.0, and 7% PVPP. At the end of the 24-hr feeding, the regurgitate of these same insects was obtained by gently holding the larvae behind the head with forceps and collecting regurgitated digestive fluid into microcentrifuge tubes. Fluid (ca. 500 μ l/treatment) was held on ice and mixed with 1 ml of 0.1 M potassium phosphate buffer, pH 8.0, containing 7% (w/v) PVPP. The enzyme solutions for both feces and regurgitate were centrifuged at 15,000 g for 15 min and assayed

immediately. The assay was otherwise identical to that described for foliar PPO. The POD activity was determined with POD-specific substrate guaiacol in the absence of exogenous H_2O_2 . PPO activity of the foliage fed to larvae was also determined using the potassium phosphate buffer at pH 8.0.

Determination of Effect and Midgut Enzymes on PPO Activity of Foliage. To determine if the midgut enzymes of *H. zea* affect foliar PPO activity, we incubated homogenized foliage with midgut tissue from larvae. Midguts were prepared as described by Broadway and Duffey (1986a) and added to ca. 1 g wet weight of tomato foliage (four small leaflets). Foliage was homogenized for PPO assay as described above. Three concentrations of midgut tissues were used: 18.75, 37.5, and 75.0 mg midgut wet weight per 250 mg foliage wet weight and including a control with no midgut tissue. Additionally, a midgut preparation containing 75 mg midgut was heated to 100°C prior to adding to the PPO preparation as a control. The midgut and PPO solution was incubated at 25°C for 15 min prior to PPO determination. A total of five replicates per treatment was completed.

Determination of Oxidation of Chlorogenic Acid During Insect Feeding. Chlorogenic acid ($[^3H]CHA$) was used to determine if it was oxidized when larvae fed upon tomato foliage. The isotope was prepared and purified as described by Isman and Duffey (1983). Sixth-instar *H. zea* or *S. exigua* were removed from artificial diet and starved for 3 hr. Leaflets of greenhouse-grown *L. esculentum* var. Ace 55 were excised from plants at the six to eight-leaf stage and transported on ice to the laboratory. Paired leaflets were collected so that one leaflet could be used for phenolic assay while the opposite leaflet could be used in the feeding experiment. The phenolic concentrations within each pair did not significantly differ when 25 pairs were examined ($P > 0.05$). This allowed us to estimate the chlorogenic acid concentration in each leaflet fed a larva so that the specific activity of the isotope could be determined for each leaflet.

Leaflets were removed from ice, one of the paired leaflets was placed in 5 ml of 50% aqueous methanol for 24 hr at 60°C, its phenolic concentration was determined as above. An 8- μ l sample of $[^3H]CHA$ in 75% aqueous methanol (specific activity of 0.7 mCi/mmol) was added to each leaflet and allowed to evaporate. Leaflets were weighed and fed to larvae. Only larvae that consumed the entire leaflet within 8 hr were used in the experiment. Four hours after ingestion of the leaflet, larvae were placed on ice, and 2.5 μ l of hemolymph was extracted from a proleg and used for estimation of chlorogenic acid after thin-layer chromatography (TLC) separation of chlorogenic acid (Isman and Duffey, 1983). Feces were pooled for each larva and immediately placed in 100% methanol at 60°C for 24 hr.

The feces were pelleted from the methanol by centrifugation at 11,000g for 10 min and were extracted 5X in 100% methanol until no radioactivity

remained in the supernatant. Methanolic extracts were pooled for each larva, evaporated in vacuo, and reconstituted in 0.1 ml methanol. The fecal pellet was extracted $3 \times$ in 2 ml 8 M urea. Urea extracts were pooled for each larva. An aliquot of the methanol and urea extracts was used for two-dimensional TLC with cellulose plates using 2% formic acid followed by *n*-butanol-ethanol-2% NH_4OH (6:1:3) as solvents. Unbound or free chlorogenic acid was extracted primarily in the methanol treatment (>90%) and had R_f values of 0.70 and 0.81 in 2% formic acid solvent (*cis* and *trans* isomers) and an R_f value of 0.95 in the butanol-ethanol- NH_4OH solvent. To determine if CHA was covalently bound to protein and/or amino acids, different replicates of the cellulose plates were sprayed with reagents for phenolics (0.5% diphenylborate for orthodihydroxyphenols, and Folin-Ciocalteu reagent for phenols; Harborne, 1982) and with ninhydrin for primary amines (Harborne, 1982). The bands were scraped from the TLC plates, eluted with the appropriate solvent, and mixed with aqueous scintillation fluid (ACS, Amersham Corporation, Arlington Heights, Illinois). Total radioactivity in each sample was determined by the channel ratios method in a Beckman LS 230 liquid scintillation counter.

Phenologic and Species Differences in Phenolics and PPO. In order to examine how much phenolic content and PPO activity vary over time and between varieties and species in the field, we grew 25 plants of the following during the summer of 1985: *L. esculentum* var. Castlemart; *L. esculentum* var. Walter; *L. hirsutum* f. glabratum LA 2100, and *L. hirsutum* f. glabratum PI 134417. The plants were transferred from the greenhouse to the field and grown there as described. Two fully expanded, terminal leaflets were carefully excised from each plant from three different plant stages: flowering, green fruit, and mature fruit. The measurement of *o*-dihydroxyphenols and PPO from individual leaflets were as described above.

RESULTS

Substrate Specificities of Foliar Oxidases. The majority of chlorogenic acid oxidation in tomato foliage is due to PPO and not due to POD or laccase activity (Figure 1). The oxidation of hydroquinone, a substrate for laccase, was not observed in our assays. CHA is a substrate for POD as shown by a 31% increase in the rate of CHA oxidation when H_2O_2 was added. The diphenol, caffeic acid, was also a substrate for PPO/POD. However, the POD substrate, guaiacol, was oxidized at a very slow rate in the absence of exogenous H_2O_2 . Thus, endogenous levels of H_2O_2 appear to be insufficient for POD to be a major factor in the oxidation of CHA or caffeic acid during tissue damage. Tomato PPO/POD showed little or no monophenolase activity when tyrosine, *p*-coumaric acid, and *p*-hydroxybenzoic acid were used as substrates. Henceforth, the oxidation of

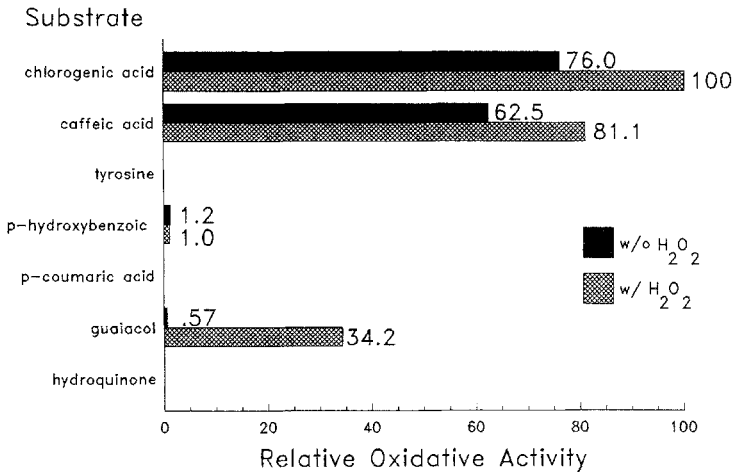


FIG. 1. The oxidative activity of tomato foliage towards various phenolic substrates. The absence of horizontal bars indicates no detectable activity.

chlorogenic acid will be referred to as PPO activity because of comparatively low POD activity in the absence of exogenous H₂O₂ and the absence of laccase activity.

Bioassays with Foliage and Fruit. The larval growth rate of *S. exigua* was reduced by more than 45% when larvae fed on diet made from foliage containing active polyphenol oxidase (Table 1). The polyphenol oxidase activity was inhibited by greater than 95% when the PPO inhibitors were added during maceration of the foliage. Additionally, in the treatment with oxidation, the measurable concentration of *o*-diphenols was reduced by over 48% and the rel-

TABLE 1. EFFECT OF POLYPHENOL OXIDASE ACTIVITY IN TOMATO FOLIAGE ON GROWTH RATE OF *Spodoptera exigua*^a

Treatment	RGR	PPO	Free NH ₂	[<i>o</i> -Diphenols]
+ Oxidation	0.215a	38.6a	0.92a	.071a
- Oxidation	0.396b	1.6b	1.10b	.138b

^aRGR = relative larval growth rate as mg/day/mg larva; PPO = polyphenol oxidase activity expressed as ΔOD₄₇₀/min/g foliage; free NH₂ = foliar concentration of free amines after treatment, expressed as percent wet weight; *o*-diphenols = foliar orthodihydroxyphenol concentration following treatment, expressed as percent wet weight. Means not followed by same letter (in columns) are significantly different at P < 0.05 following Jones (1984).

ative levels of free NH_2 groups in the amino acid extract (80% ethanol) were reduced by 16% (Table 1). A 37% reduction in Biorad-detectable protein occurred in the treatment with oxidation (data not shown). These data indicated that nearly 50% of the dihydroxyphenolics had been oxidized and that statistically significant changes in protein and amino acids occurred. In contrast, the concentrations of protein and *o*-diphenols prior to oxidation were not significantly different ($P > 0.05$) between treatments. The levels of *o*-diphenols and protein in both treatments were 2.44–2.47 $\mu\text{mol/g}$ and 3.70–3.76% fresh wt, respectively, as determined from samples of leaves taken before maceration.

In the experiments with leaflets from greenhouse plants, the relative growth rates (RGR) of *H. zea* larvae declined by nearly 50% as the plant matured (Table 2). This reduction in larval growth rate was significantly correlated with both PPO activity and rutin concentration ($P < 0.01$) but not with chlorogenic acid concentration. The variability in chlorogenic acid concentration over the three plant stages examined was smaller than the variability in rutin. Bioassays with artificial diet indicate that these changes in chlorogenic acid concentration were too small to produce observable changes in RGR (unpublished data).

The experiments with field grown plants helped us to investigate the relationship between foliar PPO and larval RGR at different stages of plant maturity. A significant correlation between PPO and RGR was found within three of the stages examined: flowering ($r = -0.496$, $N = 99$, $P < 0.01$); immature green fruit ($r = -0.609$, $N = 100$, $P < 0.001$); and mature green fruit ($r = -0.323$, $N = 50$, $P < 0.05$). The relationship between PPO and RGR was not significant when plants with ripe fruit were examined ($r = -0.208$, $N = 70$, $P > 0.05$). In the case of the plants with mature green fruit, the PPO activity was over 3X higher than at any other stage, and consequently, most larvae were

TABLE 2. EFFECT OF PLANT STAGE OF GREENHOUSE-GROWN TOMATO PLANTS ON GROWTH RATE OF *Heliothis zea* LARVAE^a

Plant stage	RGR	N	PPO	[CHA]	[Rutin]
Preflowering	0.316	16	12.0	3.58	1.84
Flowering	0.202	15	14.8	4.87	3.89
Green fruit	0.159	15	19.8	4.42	6.83
Regression coefficient (r)			-0.55 ^b	-0.13	-0.43 ^b

^aRGR = relative larval growth rate (mg/day/mg larva); PPO = foliar polyphenol oxidase activity as $\Delta\text{OD}/\text{min}/\text{g}$; [CHA] or [rutin] = foliar concentration of chlorogenic acid or rutin expressed as $\mu\text{mol/g}$ foliage wet weight. Regression coefficient (r) = coefficient from the regression of RGR with PPO, CHA, or rutin.

^bSignificant coefficient at $P < 0.01$.

unable to grow. A logarithmic transformation of PPO activity provided the best fit of the data using the equation $y = a + b(\ln x)$. The overall regression coefficient for samples from all plant stages was -0.417 ($N = 319$, $P < 0.001$). To determine if the PPO activity reduced the rate of food consumption by larvae and hence RGR, we calculated the relative consumption rate (RCR) following Waldbauer (1968). There appeared to be no relationship between RCR and PPO and RGR: the correlation coefficient of RCR with PPO was $+0.201$ ($N = 50$, $P > 0.05$). This agrees with results obtained from bioassays using CHA + PPO in artificial diets (Kreisfeld, 1987).

The mean values of PPO and RGR for each stage of plant development examined are plotted in Figure 2, which includes the experiments with both greenhouse and field plants. A correlation between the mean PPO and RGR values was highly significant ($r = -0.953$, $P < 0.01$). The RGR of larvae was also correlated with polyphenol oxidase activity in fruit (Figure 3). The PPO activity of fruit is much lower than foliage and virtually ceases by the time the fruit matures. The RGR of larvae on maturing fruit (0.37–0.47) was faster than on foliage from any of the stages examined (0.316, the fastest). The correlation between PPO activity and RGR on fruit was significant ($r = -0.811$, $N = 30$, $P < 0.01$).

Bioassays with Artificial Diet. The bioassays with CHA + PPO added to artificial diet indicated that the polymer formed during oxidation of CHA in the absence of competing $-\text{NH}_2$ or $-\text{SH}$ groups was not toxic to *S. exigua* larvae at the levels tested (Table 3). However, when CHA + PPO were allowed to

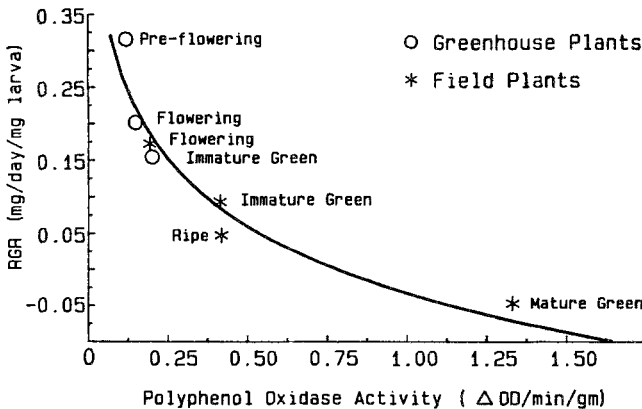


FIG. 2. The relationship of foliar polyphenol oxidase activity to the relative growth rate of *Heliothis zea* at different plant stages. The regression equation was $y = \ln(0.968) - 0.134(\ln x)$; $r = -0.953$, $N = 6$. This regression was derived from the mean values obtained for each plant stage from greenhouse plants and field plants. PPO activity expressed as $\Delta\text{OD} \times 10^{-2}/\text{min/g}$ foliage.

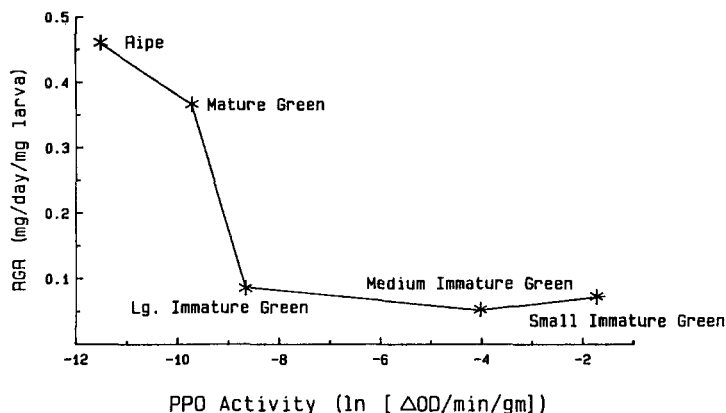


FIG. 3. The relationship of fruit polyphenol oxidase activity to the relative growth rate of *Heliothis zea* at different stages of fruit maturity. The regression equation was $y = \ln(0.940) - 0.081(\ln x)$; $r = -0.811$, $N = 30$. The equation was obtained from the regression of the individual larval growth rates with the mean polyphenol oxidase activity of fruit for each stage.

react in the presence of casein protein or the casein digest, a substantial reduction in larval weight occurred. These data indicate that the reduction in larval growth by CHA + PPO occurred as a result of its interaction with protein or free amino acids and was not due to any direct toxic properties of the polymer. In contrast, CHA in its reduced state was not toxic to larvae at a low level of

TABLE 3. EFFECT OF OXIDATION OF CHLOROGENIC ACID (CHA) IN ARTIFICIAL DIET ON *Spodoptera exigua*

Treatment	Relative growth ^a
0.5% casein	
Control	100.0a
CHA	102.0a
CHA + PPO (polymer) ^b	90.6a
CHA + PPO (alkylated casein)	44.5b
0.5% casein digest ^c	
Control	100.0a
CHA	27.9b
CHA + PPO (alkylated amino acids)	37.5b

^aRelative growth = weight of larvae on test diet divided by weight of larvae on control diet × 100.

^bPolymer formed by oxidation in absence of casein.

^cCasein digest = casein digested by bovine protease to TCA-soluble peptides and free amino acids.

casein (0.5% w/w) but was considerably more toxic in diets containing tryptically digested casein (0.5%). Unpublished data indicate that the toxicity of CHA is enhanced by increasing levels of free amines. The concentration of CHA used in the bioassays (3.5 $\mu\text{mol/g}$ wet weight) was the approximate mean concentration of CHA in Castlemart variety tomato plants during a preceding growing season (Felton et al., 1987).

Amino Acid Analyses. The amino acid analyses of isolated tomato protein incubated with active tomato PPO or with tomato PPO inactivated by PTU, indicate significant changes in amino acid composition between treatments (Table 4). When protein is incubated with an active preparation of tomato PPO (which also is contaminated with ca. 0.7% free amino acids), nearly all of the amino acids were reduced by over 11% except for glutamic acid. The overall reduction in identifiable free and proteinaceous amino acids was 14.4%. However, the experiments with tomato protein and purified mushroom tyrosinase as

TABLE 4. EFFECT OF OXIDATION OF CHLOROGENIC ACID ON AMINO ACID CONTENT^a OF TOMATO PROTEIN AND FREE AMINO ACIDS

Amino acid	Tomato protein + free amino acids ^b		Tomato Protein ^c	
	- Oxidation	+ Oxidation	- Oxidation	+ Oxidation
ASP	20.99	17.95	42.71	42.51
THR	9.76	8.18*	11.67	11.56
SER	11.29	9.32*	13.31	13.12
GLU	23.99	22.00	32.61	33.32
PRO	16.46	13.44*	18.46	18.46
GLY	24.13	20.30*	30.08	29.62
ALA	21.88	18.40*	26.45	26.68
VAL	19.69	16.35*	24.62	24.76
MET	4.48	3.60*	5.54	5.42
ILE	13.31	11.13*	17.07	16.94
LEU	21.31	17.90*	27.39	27.08
TYR	8.05	6.77*	12.00	10.93
PHE	10.73	9.06*	17.00	14.63
HIS	3.91	3.19*	5.04	4.72
LYS	11.89	9.92*	14.63	13.47
ARG	7.14	6.30	8.31	8.12

^a Amino acid content expressed as mM/100 g sample. An asterisk denotes differences between oxidation treatments greater than 15%.

^b Treatment contained chlorogenic acid, tomato foliar protein, and tomato PPO containing soluble protein and free amino acids. PPO inhibitors added to - oxidation treatment prior to reaction.

^c Treatment contained chlorogenic acid, tomato protein, and purified mushroom tyrosinase for PPO activity. PPO inhibitors added to - oxidation treatment prior to reaction.

PPO showed little difference in amino acid compositions (Table 4). The most significant changes in the latter tests were a 14% reduction in phenylalanine and an 8% reduction in each of lysine and tyrosine.

In contrast to the changes in amino acid composition that occurred as a result of phenolic oxidation, the measurable percent of Kjeldahl nitrogen was not affected by oxidation. The treatment containing tomato protein and active tyrosinase had 78.3% Kjeldahl protein N (% protein = $N \times 6.25$), whereas the treatment with inactivated tyrosinase had 78.4%. In the treatments containing tomato PPO, the Kjeldahl protein N was 47.3% for treatments containing either active or inactivated PPO.

Determinations of Polyphenol Oxidase Activity in Regurgitate and Feces. Significant PPO activity exists in the larval mid- and hindgut when larvae are fed tomato foliage (Table 5). However, no PPO activity could be detected in the regurgitate or the feces of larvae fed artificial diet. The activity of PPO in the regurgitate of foliage-feeding larvae was 66.7% of that in the ingested foliage, whereas the feces produced by these larvae had PPO activity significantly higher than the foliage. No POD activity with guaiacol substrate could be detected in regurgitate or feces in the absence of exogenous H_2O_2 . These results indicate that the tomato PPO is not inhibited or neutralized by the insect gut, and do suggest that factors in the gut may increase PPO activity as the food bolus passes through the digestive system.

Effect of Larval Midgut on PPO Activity. When homogenates of tomato foliage were incubated with larval midgut tissue, a significant increase ($P < 0.01$ with $r = 0.91$) in foliar PPO activity of nearly 40% was observed (Figure 4). When midgut tissue was heated to inactivate digestive enzymes, no increase in PPO activity was observed. These results suggest that tomato PPO activity is not reduced as a consequence of midgut proteases but that PPO activity is

TABLE 5. POLYPHENOL OXIDASE (PPO) ACTIVITY IN REGURGITATE AND FECES OF *Heliothis zea* FED TOMATO FOLIAGE OR ARTIFICIAL DIET

Source of PPO	Relative activity ^a
Plant foliage	100.0a
Larval regurgitate	
Foliage	66.7b
Artificial diet	0.0c
Larval feces	
Foliage	113.0d
Artificial diet	0.0c

^aRelative polyphenol oxidase activity expressed as percent of control as $\Delta OD_{470}/\text{min}/\text{mg}$ dry weight. Control = PPO activity of the ingested foliage. Means not followed by the same letter are significantly different at $P < 0.05$ (Jones, 1984).

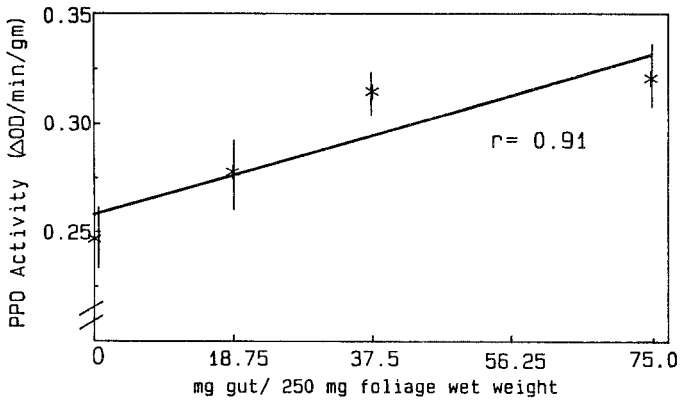


FIG. 4. The effect of *Heliothis zea* midgut tissue on tomato foliar polyphenol oxidase activity. One midgut = 75.0 mg with a tryptic activity of 35 nmol *p*-TSA/min/mg gut after Broadway and Duffey (1986a). Error bars represent 95% confidence limits with $N = 4$ determinations per midgut concentration. PPO activity expressed as $\Delta OD \times 10^{-2}/\text{min/g foliage}$.

activated by these enzymes. Additionally, no PPO activity (as chlorogenic acid oxidase) could be detected in the midgut homogenates used in the assay. POD activity was absent in all assays in absence of exogenous H_2O_2 .

Effect of pH on Tomato Foliar PPO. Tomato foliar PPO is active across a wide pH range (Figure 5). Optimal activity occurs at pH 6.8, but substantial

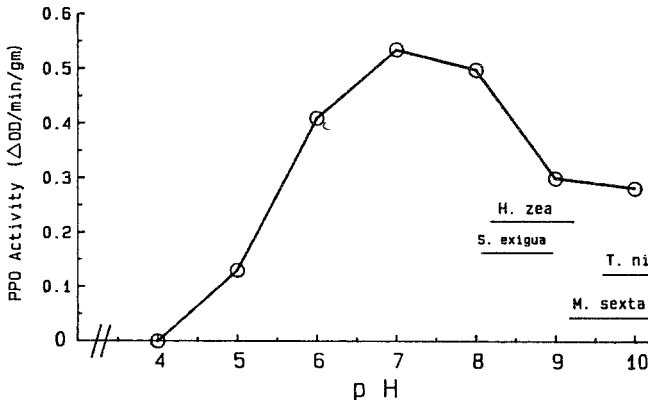


FIG. 5. The effect of pH on tomato foliar polyphenol oxidase activity. The horizontal bars indicate the pH range of the midguts of the species shown: *Trichoplusia ni*, *Manduca sexta*, *Heliothis zea*, *Spodoptera exigua*. Midgut pHs are from Broadway (1985), J.S. Martin et al. (1987) and unpublished data. PPO activity expressed as $\Delta OD \times 10^{-2}/\text{min/g foliage}$.

activity is present from 6.0 to 10.0. The midgut pH values of four lepidopterous pests of the tomato plant appear in Figure 5. All four pest species—*Manduca sexta*, *Trichoplusia ni*, *S. exigua* and *H. zea*—have gut pH values within the range of PPO activity. In fact, the gut pH of the first two species *M. sexta* and *T. ni*, is near 10.0, a pH concentration at which CHA will autoxidize to form the quinone (Barbeau and Kinsella, 1983).

Experiments with [³H]Chlorogenic Acid. The results we obtained with the isotope are summarized in Table 6. When both *S. exigua* and *H. zea* larvae fed on artificial diet containing chlorogenic acid, ca. 90% of the excreted chlorogenic acid was found to be in a free form rather than bound to amino acids or protein. However, when larvae were fed tomato foliage, much less of the ingested chlorogenic acid was free and significant amounts were bound to protein: nearly 50% of the excreted chlorogenic acid was bound to protein for *H. zea* larvae and nearly 40% for *S. exigua*. These results do not necessarily suggest that there are species differences, however, because the foliage ingested by *H. zea* larvae was substantially higher in PPO activity than that ingested by *S. exigua* (19.3 OD₄₇₀/min/g foliage vs. 12.0).

The amount of chlorogenic acid absorbed into the hemolymph was not significantly different for *H. zea* larvae ingesting either artificial diet or tomato foliage (Table 6). However, *S. exigua* larvae ingesting tomato foliage showed slightly higher levels of uptake than those fed artificial diet. More than 85% of the recoverable radioactivity in the hemolymph samples occurred as free chlorogenic acid. Less than 10% of the radioactive chlorogenic acid was bound to protein for either species.

Excreted chlorogenic acid in the free form was obtained primarily in the methanolic extracts of the feces. A brownish phenolic- and amino-positive material was detected in the urea extracts with an $R_f = 0.70-0.96$ in formic

TABLE 6. UPTAKE AND EXCRETION OF [³H]CHLOROGENIC ACID (CHA) BY NOCTUID LARVAE^a

	<i>Heliothis zea</i>	<i>Spodoptera exigua</i>
Percent ingested CHA in hemolymph		
Foliage	5.2(0.8)	7.9(0.9)
Artificial diet	3.6(1.0)	5.3(0.6)
Percent of excreted CHA bound to protein		
Foliage	49.0(4.1)	38.3(3.8)
Artificial diet	4.5(0.6)	6.0(1.2)
Percent of excreted CHA as unbound		
Foliage	45.0(6.2)	56.6(4.4)
Artificial diet	90.2(4.5)	93.0(5.6)

^a Values are means for $N = 10$ per treatment. Numbers in parentheses are 95% confidence intervals.

acid and $R_f = 0.00-0.02$ in NH_4OH solvent. This material contained radioactivity from the labeled CHA and reacted with all reagents used for the detection of phenolics and amines. Additionally, it reacted strongly with Coomassie blue reagent for the detection of proteins (Bradford, 1976). Furthermore, a purification process consisting of elution from the cellulose, dialysis (6000 mol wt cutoff) against 8 M urea to dissociate hydrogen-bonded CHA, and dialysis against ddH_2O failed to alter the effect of the reagents on the material or to remove any significant amount of radioactivity. Several properties of the material indicated the presence of chlorogenic acid covalently bound to protein: it was insoluble in 10% TCA and 100% methanol, showed very little mobility in NH_4OH solvent, tested positive with phenolic and amine reagents, contained radioactivity, and was not dialyzable in 8 M urea at a 6000 mol wt. The phenolic-protein material was then saponified with 2 N NaOH for 24 hr at 2°C , a treatment known to hydrolyze the quinic acid ester of chlorogenic acid when bound to protein (Davies et al., 1978). Following saponification, quinic acid was detected on TLC and compared to a standard (Davies et al., 1978). The released quinic acid contained radioactivity associated with the $[^3\text{H}]\text{CHA}$. Moreover, a similar material was extracted from foliage that had been macerated and allowed to oxidize for 1 hr, but no comparable material was obtained from intact foliage that had been heated at 80°C for 2 hr prior to maceration and extraction. Finally, 5–10% of the radioactivity of extracts from foliage-derived feces was associated with a phenolic-positive material that remained near the origin in both TLC solvents. This material was not amine positive and may represent the polymer formed during the oxidation of CHA. A similar material is produced when PPO (mushroom tyrosinase) and CHA are incubated in the absence of any other additional protein. Experiments are under way to characterize the chemical properties of these materials. Approximately 80% of the total ingested radioactivity was recoverable in the methanol and urea feces extracts in all treatments.

Phenological and/or Varietal Changes in PPO and Phenolics. Foliar PPO activity significantly increased during plant maturation for all four plant species and varieties examined (Figure 6). The greatest PPO activity occurred within the two commercial varieties of *L. esculentum*, Castlemart and Walter, whereas consistently lower values occurred in *L. hirsutum* species. These results were obtained from 1985 and differed slightly from those obtained in 1987 with the Castlemart variety. PPO activity was highest at the mature green fruit stage during 1987 and lower at the ripe stage. In 1987, the last sampling date was approximately one month later than in 1985 (October vs. September). Reductions in PPO activity may be a consequence of the lower temperature and/or reduced solar radiation. Foliage also contains peroxidase activity (not reported here) that has chlorogenic acid oxidase activity but with only 20–25% of the activity of PPO.

In contrast to the above findings with PPO, levels of phenolics remained

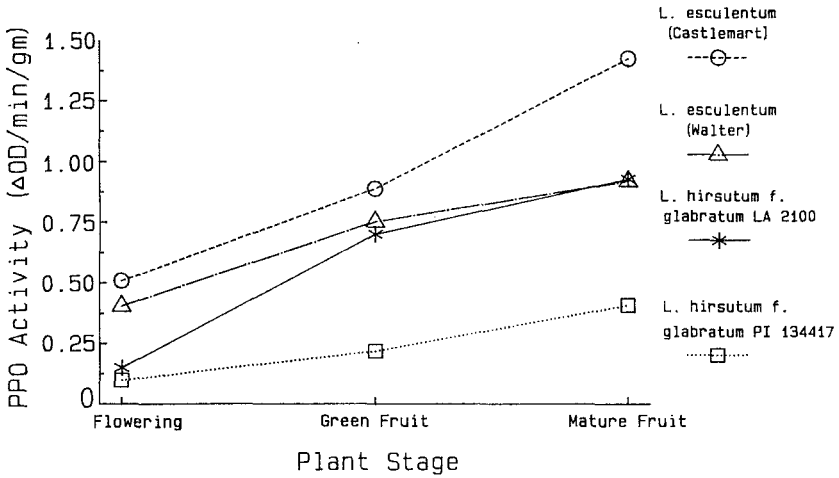


FIG. 6. The effect of plant stage on foliar polyphenol oxidase activity in four varieties or species of *Lycopersicon*. PPO activity expressed as $\Delta OD \times 10^{-2}/\text{min/g}$ foliage.

fairly stable in most species or varieties with the exception of *L. hirsutum* f. *glabratum* LA2100, which showed a significant decline in phenolic concentration during plant maturation (Figure 7). The levels of phenolics were generally higher in *L. hirsutum* species than in commercial species *L. esculentum*.

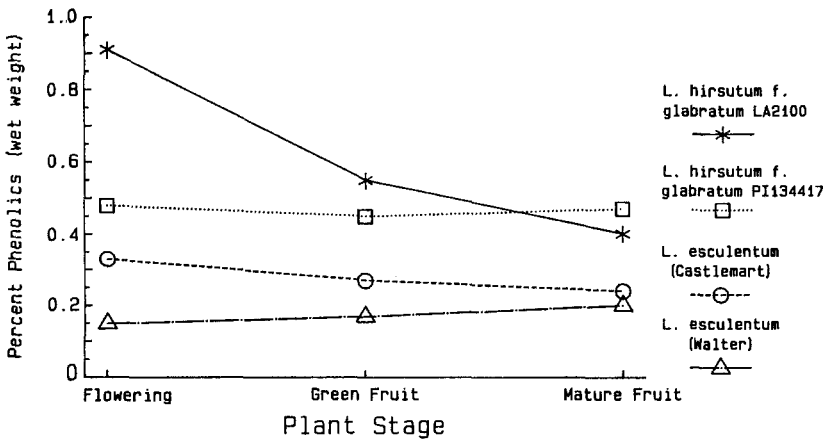


FIG. 7. The effect of plant stage on foliar phenolic levels in four varieties or species of *Lycopersicon*.

DISCUSSION

A significant amount of ingested chlorogenic acid was oxidized by foliar PPO when noctuid larvae fed on tomato foliage. Apparently, the greatest majority of CHA is oxidized by PPO and not by laccase or POD, which are either absent from or at very low activity in foliage (Figure 1). Extracts of foliage failed to oxidize the laccase substrate, hydroquinone, and showed little activity against guaiacol, a peroxidase substrate, unless exogenous H_2O_2 was added. Also POD could not be detected in insect regurgitate, midgut, or feces in the absence of exogenous H_2O_2 . We have preliminary data that indicates that H_2O_2 is rapidly eliminated in ingested plant tissues by catalases of plant and insect gut origin.

Other studies have reported the presence of PPO activity in the digestive systems of insects (e.g., foregut of a grasshopper; Rhoades, 1977; and salivary glands of Hemiptera; Miles, 1978). Rhoades (1977, 1983) speculated that the PPO in these insects may be an adaptation to neutralize the defensive properties of plant phenolics. In marked contrast, our data indicated that PPO activity substantially reduced larval growth rates and that activity present in the gut was derived from ingested foliage.

The reduction in larval growth in bioassays with oxidized tomato foliage (Table 1) or artificial diets (Table 3) may be due to one or more of the following mechanisms: first, reduction in the bioavailability of amino acids via direct binding of the chlorogenoquinone to $-NH_2$ and/or $-SH$ groups of free amino acids and proteins (Hurrell et al., 1982; Igarashi and Yasui, 1985); second, loss of free amino acids via the enzymatic degradation of free amino acids to aldehydes in the presence of PPO and phenolic substrates (Motoda, 1979); third, reduced digestibility of dietary protein (Free and Satterlee, 1975; Barbeau and Kinsella, 1985); and fourth, potential inhibition of insect digestive enzymes (G.W. Felton, unpublished data). Substantial reductions in foliar free amino acids occurred when a preparation of tomato PPO (containing free amino acids) oxidized chlorogenic acid (Table 4), suggesting that the bioavailability of free amino acids may be severely reduced to herbivores feeding on foliage rich in PPO and diphenol substrates. Although minor changes occurred in the amino acid composition of tomato protein in the presence of PPO (purified mushroom tyrosinase) and CHA, it is known that alkylation of the *E*- NH_2 moiety of lysine reduces the digestibility of protein (Hurrell et al., 1982; Pierpoint et al., 1977). We observed an 8% reduction in the measurable lysine of tomato protein treated by CHA and PPO (Table 4), indicating a loss of available lysine, which may contribute to reduced protein digestibility. Other researchers have documented the reduced *in vitro* digestibility of leaf protein alkylated by oxidized chlorogenic acid (Barbeau and Kinsella, 1985). Our data from bioassays with artificial diet indicated that the adverse effects of chlorogenic acid oxidation occurred as

a result of the covalent interaction between the generated quinone and dietary protein (or free amines) and were not due to the polymerization of the phenolic in the absence of reactive SH or NH₂ groups (Table 3). The CHA-amine product isolated from the feces, although having a molecular weight greater than 6000, does not exclude the possibility that chlorogenoquinone binds to free amino acids in the insect gut because the product formed may be a result of secondary reactions involving intermolecular cross-linking of amino acids, peptides, and proteins (Pierpoint, 1983; Matheis and Whitaker, 1984).

Our results may be extrapolatable to other plant-insect systems and suggest that the oxidation of phenolics may contribute significantly to reducing the nutritive quality of foliage. Polyphenol oxidases are widespread among angiosperm and gymnosperm plant species and cooccur with *o*-diphenol substrates (Mayer and Harel, 1979; Mayer, 1987). The oxidation of phenolic compounds and their subsequent polymerization on the leaf surface via damaged trichomes has been identified as a defense mechanism against small sucking insects such as aphids or leafhoppers (Gregory and Tingey, 1981; Gregory et al., 1986; Duffey, 1986). Rhoades (1977) showed that the growth rate of the orthopteran *Astroma quadrilobatum* was negatively correlated with polyphenol oxidase activity in the creosote bush *Larrea cuneifolia*.

The antibiotic effect of oxidized phenolics may be enhanced by the normal gut conditions of these insects. For example, at pHs lower than 6.5, the alkylation of free amino acids by chlorogenoquinone apparently does not occur (Haider et al., 1965), but at alkaline pH (ca. pH 10), the oxidation of chlorogenic acid can occur both nonenzymatically and enzymatically via polyphenol oxidase or peroxidase (Barbeau and Kinsella, 1983; Pierpoint, 1983). The gut pH of most lepidopterous larvae exceeds 6.5 (Berenbaum, 1980) and sometimes even 10.0, as in the case of a specialist folivore of tomato, *Manduca sexta* (J.S. Martin et al., 1987), thus the potential for oxidation appears greater for these insects than other insects such as acridids whose gut pHs may often lie at or below 6.0 (Bernays, 1981). The gut pH of both *H. zea* and *S. exigua* is within the range for activity of tomato PPO (Figure 4). Characteristically, ranges of pH activity for plant polyphenol oxidases are quite broad (Mayer, 1987), suggesting that these enzymes are active in the gut of many insects and may present a formidable barrier against selection for resistance by herbivores via changes in midgut pH. Furthermore, our data indicate insect proteolytic enzymes may activate latent or insoluble membrane-bound PPO activity. Because of the ubiquitous cooccurrence of PPO and diphenols among plant species and the potential for PPO activity in insect guts, this potential mechanism of digestibility reduction may be more applicable to plant defense theories (e.g., Rhoades and Cates, 1976; Feeny, 1976; Coley et al., 1985) than are the digestibility-reducing properties previously ascribed to tannins but now strongly contested (Bernays, 1981; J.S. Martin et al., 1987).

Many herbivores have higher growth rates, fecundity, and survival on young leaves than on senescing leaves (White, 1984; Raupp and Denno, 1983). In many plants, including tomatoes, the level of PPO activity increases with leaf or plant age (Figure 7) (Mayer and Harel, 1979; Mayer, 1987) while the highest levels of foliar antioxidants occur in young leaves (e.g., ascorbic acid, glutathione, tocopherols, and carotenoids). Thus, the greater potential for phenolic oxidation in mature foliage may be one reason why insect growth sometimes correlates with the phenology of foliage. The larvae of both *S. exigua* and *H. zea* migrate to tomato fruit at an early instar (Lange and Bronson, 1981). This behavior allows the larvae to feed on a food source with higher water content and to escape mortality from parasites, predators, and pathogens on the exposed leaf surfaces. Another selective advantage for this feeding behavior could be the avoidance of toxic levels of PPO activity and of phenolics. Green or mature fruit contained significantly less PPO activity than foliage, as measured in our studies (Figure 3) and others (Rhodes and Wooltorton, 1977). In addition, the fruit levels of substrates (e.g., chlorogenic acid) for this enzyme significantly decline with fruit development (Wardel, 1973). Consequently, larvae migrating from foliage to the maturing fruit not only can feed in a protected microenvironment but may also avoid the antibiotic effects of these phytochemicals.

Many studies indicate that nitrogen is a limiting factor for herbivore survival (White, 1984; Mattson, 1980; Scriber and Slansky, 1981 and references therein), but most have relied solely upon the estimate of foliar nitrogen by the Kjeldahl procedure. Here, the oxidation of chlorogenic acid by PPO did not result in any significant change in total Kjeldahl nitrogen (measures all N liberated as NH_3) of tomato protein despite striking changes in amino acid composition induced by alkylation (Table 4). The alkylation of protein or free amino acids by chlorogenoquinone appears to reduce the quality of the foliage as a food source for larvae (Table 3). In instances where herbivores consume foliage rich in PPO and *o*-diphenols, or other plant chemicals that can covalently bind to protein such as lactones, allylisothiocyanate, gossypol, hydroperoxides (Kawakishi and Kaneko, 1987; Finley et al., 1973), the relationship of herbivore performance to dietary nitrogen (e.g. Scriber's physiological efficiency model (1984), may be obscured when the measurement of nitrogen is limited to the Kjeldahl procedure. Conversely, the detectability of protein by Coomassie blue reagent was reduced in tomato foliage that was allowed to oxidize after maceration. This reduction may be due to specific binding of chlorogenoquinone to lysine or histidine, which may cause changes in the aqueous solubility of foliar protein and/or interfere with the binding of the protein reagent to these amino acids (Mattoo et al., 1987). Coomassie blue reagent binds specifically to basic amino acids such as arginine, histidine, and lysine (Bradford, 1976; Compton and Jones, 1985). More rigorous procedures for estimating qualitative and quantitative changes in foliar nitrogen are needed if cause-and-

effect relationships between plant nitrogen and herbivore fitness are to be better assessed.

The oxidation of *o*-dihydroxyphenolics in the gut of insects may have other consequences in addition to the reduced capacity to utilize plant nitrogen. Because chlorogenoquinone is highly reactive and alkylates free primary amines, sulfhydryl groups, and possibly peptide bonds, other molecules containing these nucleophilic groups may be alkylated (Matheis and Whitaker, 1984; Pierpoint, 1983). We have experimental evidence that the proteinase inhibitors of the tomato plant are alkylated by oxidation of chlorogenic acid, resulting in reduced ability to inhibit trypsin and a subsequent loss of antibiotic activity to insects (Felton et al., 1989). Additionally, the protein coat of a nuclear polyhedrosis virus infecting noctuid larvae is readily alkylated by these quinones, causing substantial loss of viral infectivity in larval *H. zea* (Felton and Duffey, 1990).

It follows that the effects of oxidized phenolics on insect herbivores may be multifaceted and multitrophic: with adverse effects on nutrients through reduced utilizability of protein and free amino acid nitrogen but potentially salutary effects through reduced activity of toxins (e.g., proteinase inhibitors and nonprotein amino acids) and reduced infectivity of pathogens (e.g., nuclear polyhedrosis viruses). Further ecological and physiological investigations are needed to clarify the complex multitrophic interactions among the plant, the herbivore, and its natural enemies.

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ALARM PHEROMONE OF PENTATOMID BUG, *Erthesina fullo* THUNBERG (HEMIPTERA: PENTATOMIDAE)

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Abstract—In the pentatomid bug, *Erthesina fullo* Thunberg, the odor of male metathoracic scent gland elicits an alarm response, making the male individuals of the same species alert and disperse; the alarm response of males is more obvious than that of females. Chemical composition of the glandular secretion was identified by gas chromatography and mass spectrometry data in comparison with authentic compounds. No sexual dimorphism exists in the glandular composition in this species. A total of 9 compounds [(*E*)-2-hexenal, (*E*)-4-keto-2-hexenal, (*E*)-2-hexenyl acetate, *n*-undecane, *n*-dodecane, (*E*)-2-decenal, *n*-tridecane, (*E*)-2-decenyl acetate, and *n*-pentadecane] are identified, among which *n*-tridecane and (*E*)-4-keto-2-hexenal comprised nearly 70% of the total secretion in both females and males.

Key Words—Alarm pheromone, metathoracic scent gland, Hemiptera, Pentatomidae, *Erthesina fullo*.

INTRODUCTION

In Hemiptera, the existence of an alarm pheromone was first reported by Calam and Youdeowei (1968); (*E*)-2-hexenal and some other aldehydic scent components of fifth instar larvae of *Dysdercus intermedius* Distant had an alarm effect to both larvae and adults. Ishiwatari (1974) also indicated that (*E*)-2-hexenal had an alarm effect on three species of pentatomid bugs, *Eurydema rugosa* Motschulsky, *E. pulchra* (Westwood), and *Nezara viridula* (L.). Lockwood and Story (1985) reported that the first instar *N. viridula* use *n*-tridecane as a bifunctional pheromone which caused dispersal at high concentrations (10^0 and 10^1 individual equivalent) and aggregation at low concentration (10^{-2} indi-

vidual equivalent). Recently, the metathoracic scent gland secretion of adult *N. viridula* was found to function as an alarm pheromone and two components, (*E*)-2-hexenal and (*E*)-2-hexenyl acetate, significantly increased movement during the first minute of exposure (Lockwood and Story, 1987).

The *Erthesina fullo* Thunberg (Hemiptera: Pentatomidae) is a major pest of pine trees and nato trees (hardwood) in Taiwan. Both males and females of this species also produce a secretion from the metathoracic scent gland that causes conspecific adults to drop from a plant or scurry and fly away from the emission site. This phenomenon indicates that the secretion may function as an alarm pheromone in *E. fullo*. In this study, we examined the biological activity and chemistry of the male and female metathoracic scent gland secretions in pentatomid bugs in Taiwan.

METHODS AND MATERIALS

Insects and Gland Extracts. Adults and late fifth instars of the *E. fullo* were collected during summer from luchu pine trees, *Pinus luchuensis* Mayer, and Formosan nato trees, *Palaquium formosanum* Hayata, in a suburb of north Taiwan. In the laboratory, nato tree twigs and 10% honey solution were supplied. The insectory was kept at 27–28°C, on a 12:12 hr light–dark cycle.

The adults were disturbed by using a forceps to strike their heads, then a clean filter paper (5 × 5 mm) was placed near the orifice of the metathoracic scent gland on the ventral metathoracic surface. The discharge sprayed on the filter paper was extracted with methylene chloride. Because different numbers of filter papers of male or female extract were immersed in different volumes of methylene chloride, a total of 136 males and 108 females were extracted in 3500 μ l and 2400 μ l methylene chloride, respectively. The extract was stored under 0°C for later bioassay and chemical analysis.

Bioassay of the Gland Extract. Two hours before the bioassay, one female or male was placed in each 17-cm × 9-cm glass jar with a piece of host twig. A total of 70 such glass jars of males or females were prepared for the six different concentrations and control. During bioassay, a small amount of female and male stock extract was taken out with a syringe and solvent was added to make the dilution equal to 1 individual equivalent (IE) per 30 μ l, and then serially diluted solutions of different IE (10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , and 1 IE) were obtained. Each solution was injected with a syringe onto the inside wall of a pipet tip, and the air was expelled with a rubber bulb toward each test individual on the host plant in the glass jar. The number of individuals that dropped from the host plant or began to move away was the criterion of the biological activity. The experiment had three replicates, so a total of 30 insects

were used at each concentration. The Kruskal-Wallis one-way analysis of variance by ranks (Siegel, 1956) was used to analyze the results.

Chemical Analysis. Gas chromatography (GC) of the extract was performed on three capillary columns. A Varian 3700 GC equipped with a flame ionization detector was used with a 4-m fused silica capillary column coated with a 0.25- μm film of DB-1 phase, a 25-m vitreous silica capillary column coated with 1.5- μm film of DB-5 phase, and a 35-m fused silica capillary column coated with 0.25- μm film of Carbowax 20 M phase. Helium, at a flow rate of 30 cm/min, was the carrier gas. In the DB-1 and DB-5 column, the extract was run at 50°C for 2 min to 250°C at 5°C/min. In Carbowax 20 M, the extract was run at 60°C for 2 min to 200°C at 5°C/min.

Gas chromatographic-mass spectrometric (GC-MS) analysis was conducted using a Finnigan 4600 mass spectrometer. The GC-MS data were obtained using a 30-m \times 0.25-mm (ID) column of DB-5 phase. The column temperature was held at 50°C for 2 min to 300°C at 30°C/min using helium as carrier gas. Electron impact (EI) mass spectra were collected at 70 eV with separator and source at 150°C.

Each compound was identified by comparison of its mass spectrum with the published mass spectrum or the mass spectrum of the authentic standards (Aldrich and Yonke, 1975; Cornu and Massot, 1979; Kitamura et al., 1984; Staddon et al., 1987). Subsequently, all the compounds identified by mass spectral data were cross-checked by comparison of the GC retention of the natural product to that of an authentic standard using the three capillary columns under the same GC conditions as described above. Standards of (*E*)-2-hexenal, (*E*)-2-hexenyl acetate, *n*-undecane, *n*-dodecane, *n*-tridecane, and *n*-pentadecane were purchased from Sigma Chemical Co., (St. Louis, Missouri). (*E*)-4-Keto-2-hexenal, (*E*)-2-decenal, and (*E*)-2-decenyl acetate were supplied by J.R. Aldrich (Insect Physiology Laboratory, USDA-ARS, Beltsville, Maryland). The relative proportions of these nine compounds in the scent secretion was ascertained from GC peak-area integration.

RESULTS

Bioassay of Gland Extract. The bioassay results showed that females are not as responsive as males to the metathoracic scent extraction (Figure 1). Although the male alarm response to 1 IE female scent extract was 85.0%, no significant difference was found at all tested female concentrations (Figure 1A), and the alarm response of females to female scent extract was not-significant at all tested female concentrations (Figure 1A). The alarm response of males to 1 IE male scent extract was 100.0%, significantly different from the alarm

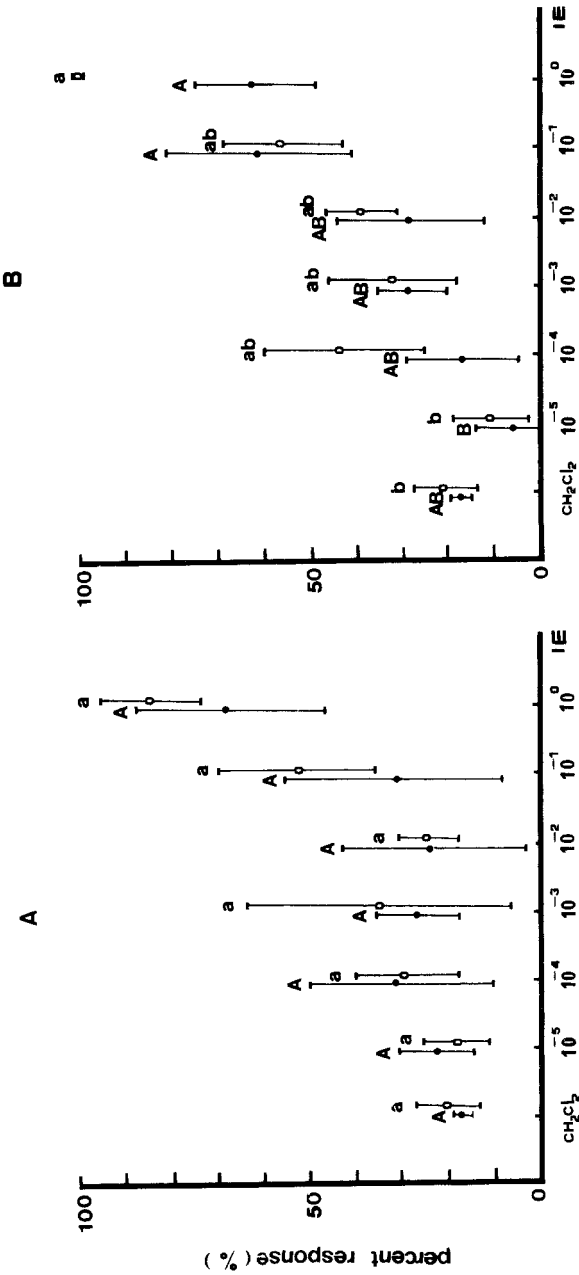


FIG. 1. Percent response of adults to different concentrations of female (A) and male (B) metathoracic scent gland extract. (●: female response, ○: male response, IE: individual equivalent). Uppercase and lowercase letters above confidence bars represent the statistical difference of females and males, respectively. Bars topped by the same letter(s) do not differ significantly; $P = 0.05$, Kruskal-Wallis one-way analysis of variance by ranks.

response to 10^{-5} male concentration ($P < 0.05$, Figure 1B); the alarm response of females to male scent extract was almost nonsignificant at all tested male concentrations (Figure 1B).

Chemical Analysis. Gas chromatographs of the metathoracic scent gland secretions of male and female are qualitatively alike (Figure 2). The secretion was composed of straight-chain alkanes [*n*-undecane (4), *n*-dodecane (5), *n*-tridecane (7), and *n*-pentadecane (9)] and α , β -unsaturated carbonyl compounds [(*E*)-2-hexenal (1), (*E*)-4-keto-2-hexenal (2), (*E*)-2-hexenyl acetate (3), (*E*)-2-decenal (6), and (*E*)-2-decenyl acetate (8)]. The mass spectral data of these compounds are listed in Table 1. The relative proportion of compounds in the scent secretion was the same in both females and males (Table 2), in which *n*-tridecane and (*E*)-4-keto-2-hexenal comprised nearly 70% of the total secretion. The other two unsaturated aldehydes, (*E*)-2-hexenal and (*E*)-2-decenal, comprised nearly 20% of the total secretion; the unsaturated acetates, (*E*)-2-hexenyl acetate and (*E*)-2-decenyl acetate, comprised nearly 8% of the total secretion; the other straight-chain alkanes only comprised nearly 2% of the total secretion.

DISCUSSION

Our work indicates that the male metathoracic scent gland secretion of *E. fullo*, functions as an alarm pheromone, which is concentration dependent, and induced obvious dispersal response at 1 IE in males. The females do not show significant alarm response to their own and male's secretion. Chemical defense against predation had been reported as the most important function of the scent glands of the pentatomids Hemiptera-Heteroptera (Blum, 1981), but in the laboratory, the phenomenon that *E. fullo* was preyed on by mantids without any defensive effect from the metathoracic gland secretion was usually observed. The biological activity of female metathoracic scent gland secretion and the reason why females are not as responsive as males still needs further study.

The metathoracic scent gland secretions of male and female *E. fullo* are nearly identical. The composition of this glandular secretion is similar to that of other pentatomids (Gilby and Waterhouse, 1965, Staddon et al., 1987). Among all identified compounds, (*E*)-2-hexenal, *n*-tridecane, and (*E*)-2-hexenyl acetate had been reported as alarm pheromones as mentioned before. Calam and Youdeowei (1968) suggested that the saturated hydrocarbons such as *n*-undecane, *n*-dodecane, *n*-tridecane, and *n*-pentadecane may function as relatively nonvolatile solvents for the active alarm pheromone components and as spreading and wetting agents in the pyrrhocorid, *D. intermedius*. Kitamura et al. (1984) also indicated that in Pentatomidae and Plataspidae hydrocarbons act as the solvent for aldehydes such as the oxohexenal. Moreover, Remold (1963),

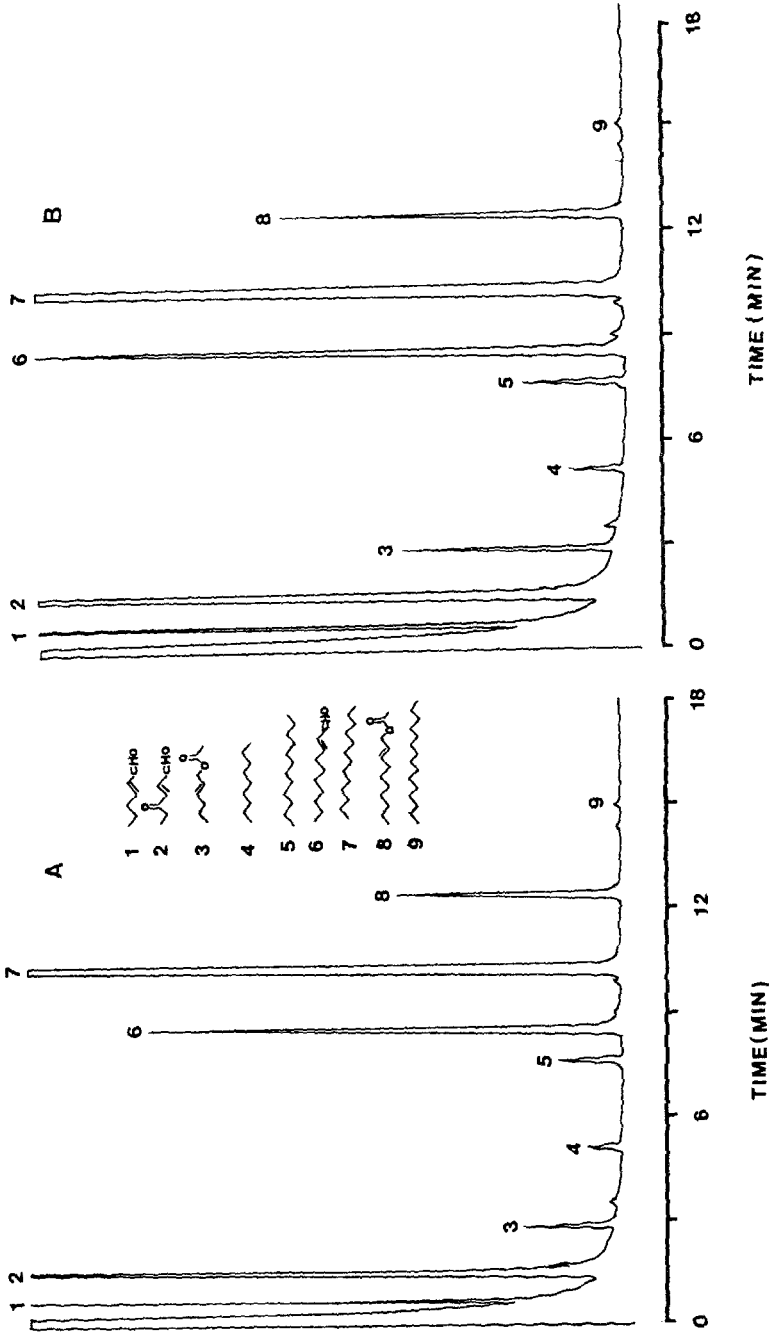


FIG. 2. Gas chromatograms of the metathoracic scent gland secretions of *Erthesina fullo* males (A) and females (B).

TABLE 1. MASS SPECTRA OF NINE COMPOUNDS IDENTIFIED IN METATHORACIC SCENT GLAND SECRETION OF *Erthesina fullo*

Compound	Mass spectral data, <i>M/Z</i> (intensity, %)
(<i>E</i>)-2-Hexenal	69(100), 55(88), 41(86), 83(80), 57(55), 42(53), 84(43), 70(42), 86(34), 43(27), 56(22), 53(16), 80(15)
(<i>E</i>)-4-Keto-2-Hexenal	83(100), 55(69), 84(24), 57(22), 43(18), 56(12), 54(8), 67(7)
(<i>E</i>)-2-Hexenyl acetate	67(100), 82(95), 84(93), 43(70), 55(58), 57(57), 86(54), 83(40), 49(34), 56(22), 100(18), 54(17), 51(16), 41(15)
<i>n</i> -Undecane	43(100), 57(90), 55(56), 41(39), 49(38), 84(33), 71(32), 83(29), 85(26), 56(25), 42(22)
<i>n</i> -Dodecane	57(100), 43(94), 71(42), 41(36), 85(33), 55(29), 56(26), 84(19), 49(18), 86(10)
(<i>E</i>)-2-Decenal	55(100), 43(70), 57(64), 41(62), 70(58), 83(48), 56(43), 69(35), 54(21), 97(20), 53(18), 67(17)
<i>n</i> -Tridecane	57(100), 43(98), 71(97), 85(73), 41(69), 56(50), 79(41), 55(34), 42(29), 84(22), 69(20)
(<i>E</i>)-2-Decenyl acetate	43(100), 54(43), 55(42), 67(35), 81(28), 68(25), 41(24), 57(23), 96(22), 82(21), 95(18), 69(17), 110(14)
<i>n</i> -Pentadecane	57(100), 43(80), 71(54), 85(42), 41(36), 55(32), 56(22), 42(15), 70(13), 99(10)

Filshie and Waterhouse (1969), and Kitamura et al. (1984) reported that many bugs possess an area of precisely elaborated cuticles surrounding the orifices of the metathoracic glands, and these structures are supposed to be the evaporative area. The present results show that *n*-tridecane and the unsaturated compounds

TABLE 2. PERCENTAGES OF COMPOUNDS IN METATHORACIC SCENT SECRETION OF FEMALE AND MALE *Erthesina fullo*

Compound	Female	Male
(<i>E</i>)-2-Hexenal	8.4	6.4
(<i>E</i>)-4-Keto-2-hexenal	21.8	19.5
(<i>E</i>)-2-Hexenyl acetate	2.4	1.6
<i>n</i> -Undecane	0.7	0.6
<i>n</i> -Dodecane	1.6	1.6
(<i>E</i>)-2-Decenal	12.1	12.9
<i>n</i> -Tridecane	46.3	50.5
(<i>E</i>)-2-Decenyl acetate	5.6	5.9
<i>n</i> -Pentadecane	0.1	0.1

such as (*E*)-4-keto-2-hexenal are the major constituents of the scent secretion of *E. fullo*. The relative proportion of (*E*)-2-hexenal was 8.4% and 6.4% in female and male, respectively, and the relative proportion of (*E*)-2-hexenyl acetate was only 2.4% and 1.6% in female and male, respectively (Table 2). Although the composition of the glandular secretion has been identified, the precise quantity and biological function of each compound in the scent secretion still need further study.

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